

# **The Effect of Incubation Time and Temperature on Sperm motility, Human Sperm DNA and Assisted Reproductive Technologies (ART) Outcome**

Estée Alwelien van Zyl

*Thesis presented in fulfilment of the requirements for the degree  
of Masters in Medical Sciences (Reproductive Biology) in the  
Faculty of Health Sciences  
University of Stellenbosch*



Supervisor: Dr Marie-Lena Windt-de Beer

March 2015

## **Declaration**

By submitting this thesis, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

**March 2015**

## ABSTRACT

In all Assisted Reproductive Technologies (ART) procedures the semen sample is handled, processed, prepared and manipulated before use in the fertilization process. During these incubation times, the sperm cells are exposed to factors that may inflict damage to the sperm structure and DNA integrity, impair its functional abilities and subsequently lead to fertilization failure and poor ART outcome. Two of the very basic, but important factors that may have an impact on the sperm quality are time and temperature exposure.

The primary objective of this study was to prospectively determine the effect of different incubation times and temperatures on motility and the DNA profile of the spermatozoa. Non-processed (n=36) and processed (n=33) semen samples were incubated for different time intervals (before: 20, 40, 60 minutes; after: 30, 60, 90 minutes) and at different temperatures (room temperature [RT] and 37°C). After incubation, sperm parameters were assessed, the CMA<sub>3</sub> assay was applied to determine chromatin maturity and compaction and the TUNEL assay to assess the level of DNA fragmentation. The results showed that in the **non-processed group**, incubation led to a time-dependent, significant decline in the motility. The highest motility was seen at 20 minutes (37°C) and motility declined in a time-dependent manner. Incubation time and temperature did not affect the CMA<sub>3</sub> and TUNEL values. Incubation of the **processed sample** led to a significant time-dependent decrease in the motility; 90 minutes (RT) had the lowest motility. The CMA<sub>3</sub> and TUNEL values between the different incubation groups did not differ significantly.

The secondary objective was to retrospectively investigate the effect of sperm incubation time **after preparation** on ART outcome. A total of 901 patient ART cycles (January 2010- December 2012) were included. Fertilization rates, embryo quality and pregnancy rates were examined. The results showed that the sperm incubation time before insemination between in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) differed significantly and the incubation time had a significant negative effect on the fertilization rates in IVF, but not in ICSI. Longer incubation times led to an unexpected significant improvement in the quality of day 2 embryos and were significantly associated with pregnancy failure in IVF and ICSI.

These combined findings suggest that non-processed semen samples can be incubated at RT or 37°C, but for no longer than 40 minutes and, for IVF, processed samples should not be incubated for longer than 60 minutes at RT or 37°C. The ICSI sample should not be incubated for more than 60 minutes although longer incubation times do not seem to influence the results for IVF. It can therefore also be concluded that sperm incubation time before insemination should be closely monitored, especially in IVF cycles.

## OPSOMMING

In kunsmatige voortplantingstegnieke (ART) word die semen-monster geprosesseer, voorberei en gemanipuleer voordat dit vir die bevrugtingsproses gebruik word. Terwyl die monster geïnkubeer word, word die spermselle blootgestel aan verskeie faktore wat die struktuur van die sperm, die DNS integriteit en die sperm se funksionele vermoë negatief kan beïnvloed. Dit kan lei tot swak bevrugting, embriokwaliteit en swangerskapsyfers. Twee basiese, maar belangrike, faktore wat die spermkwaliteit negatief kan beïnvloed is die duur van inkubasie en die temperatuur waarby die spermselle geïnkubeer word.

Die primêre doel van die huidige studie was om prospektief te ondersoek wat die effek van verskillende inkubasietype en temperature op die motiliteit en DNA profiel van die sperm het. Monsters is voor en na spermvoorbereiding vir verskillende tydsintervalle (voor: 20, 40, 60 minute; na: 30, 60, 90 minute) en verskillende temperature (kamertemperatuur [KT] en 37°C) geïnkubeer. Na elke inkubasie is 'n spermanalise, 'n CMA<sub>3</sub>- en 'n TUNEL toets gedoen. Die CMA<sub>3</sub> toets bepaal die chromatinematuuriteit en -kompaksie en die TUNEL toets vir die vlak van DNS fragmentasie. Die resultate het getoon dat daar in die **voor voorbereiding** groep 'n beduidende verskil in motiliteit tussen die verskillende inkubasiegroepes was. Die hoogste motiliteit is in die 20 minute/37°C groep gevind. Die motiliteit het oor tyd afgeneem. Die tyd en temperatuur van inkubasie het nie 'n beduidende effek op die CMA<sub>3</sub> en TUNEL uitslae gehad nie. Inkubasie **nadat** die semen **voorberei** was het weereens tot 'n beduidende verskil in motiliteit tussen die groepe gelei. Die laagste motiliteit is waargeneem by 90 minute/KT. Geen beduidende verskil is tussen die inkubasiegroepes vir CMA<sub>3</sub> en TUNEL gevind nie.

Die sekondêre doel van die studie was om retrospektief te ondersoek wat die effek van sperminkubasietyd na spermvoorbereiding op die bevrugting, embriokwaliteit en swangerskapsyfers is. 901 pasiëntsiklusse is in die studie ingesluit (Januarie 2010 tot Desember 2012). Die resultate het aangedui dat die inkubasietype van die intrasitoplasmatiese inspuiting (ICSI) en in vitro bevrugting (IVB) beduidend van mekaar verskil het. Langer inkubasietype het 'n beduidende negatiewe effek op die bevrugtinguitslae van IVB siklusse gehad, maar geen effek op ICSI siklusse gehad nie. Langer inkubasietype het ook tot 'n onverwagte verhoging in die kwaliteit van dag 2 embrios gelei en was verder beduidend geassosieer met negatiewe swangerskapuitkoms.

Hierdie gesamentlike bevindinge dui aan dat semenmonsters voor voorbereiding by KT of 37°C geïnkubeer kan word, maar nie vir langer as 40 minute nie. Na semenvoorbereiding, behoort die IVB semenmonster vir nie langer as 60 minute voor inseminasie geïnkubeer te word nie (KT of 37°C). Die ICSI semenmonster moet verkieslik binne 60 minute na voorbereiding gebruik word, maar dit wil voorkom asof die tyd hier nie so 'n groot rol speel nie. Daar kan verder afgelei word dat sperminkubasietype voor die gebruik vir inseminasie baie goed gemonitor moet word – veral in IVB siklusse.

## ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people who supported me throughout this course

- **Prof Kruger** – for giving me the opportunity to do my postgraduate studies at the University of Stellenbosch and Drs Aevitas
- **Dr Marie Lena Windt- de Beer**: For all her patience, support, guidance and immense knowledge throughout the course
- **Evelyn Erasmus** – For her guidance and her willingness to help with practical and thesis work
- **Lab personnel at Dr Aevitas** - For all their help and assistance during the two years
- **Justin Harvey and Carl Lombard** – For analyzing statistical data for this study
- **Fellow students Emma and Marlize** – for all the support, laughs, and good times we had. This experience would not have been the same without you!
- **My family** - who were always there to encourage me
- **Heavenly Father**

## Table of Contents

<b>Declaration.....</b>	<b>I</b>
<b>Abstract.....</b>	<b>III</b>
<b>Opsomming .....</b>	<b>IV</b>
<b>Acknowledgements.....</b>	<b>V</b>
<b>Table of Contents .....</b>	<b>VI</b>
<b>List of Figures .....</b>	<b>X</b>
<b>List of Tables .....</b>	<b>XII</b>
<b>Abbreviations .....</b>	<b>XIV</b>
 <b>CHAPTER 1: BACKGROUND AND LITERATURE REVIEW .....</b>	 <b>1</b>
<b>1.1. Introduction .....</b>	<b>1</b>
<b>1.2. History of IVF .....</b>	<b>2</b>
<b>1.3. Male infertility and Standard Semen Parameters .....</b>	<b>5</b>
1.3.1 Semen Physiology .....	5
1.3.1.1 Spermatogenesis.....	6
1.3.1.2 Spermiogenesis .....	6
1.3.1.3 Post-testicular sperm maturation .....	7
1.3.1.4 Seminal Plasma.....	7
1.3.2 Significance of Semen Parameters.....	8
1.3.2.1 Sperm Motility .....	8
1.3.2.2 Sperm Concentration.....	8
1.3.2.3 Sperm Morphology .....	9
1.3.3 Maturation of the sperm cells .....	10
1.3.3.1 Capacitation.....	10
1.3.3.2 Hyperactivation .....	11
1.3.3.3 The acrosome reaction .....	12
1.3.3.4 Capacitation and the acrosome reaction in vitro .....	12
<b>1.4. Semen handling and incubation .....</b>	<b>14</b>
1.4.1 Production Method.....	14
1.4.2 Container .....	14
1.4.3 Days of Abstinence .....	14
1.4.4 Exposure Time and Storage Temperature .....	14
1.4.4.1 First Incubation: Semen Collection to Sperm Preparation .....	15

1.4.4.2 Second Incubation: Sperm Preparation to ART procedure .....	16
<b>1.5. Sperm Preparation .....</b>	<b>18</b>
1.5.1 Removal of the seminal plasma .....	18
1.5.2 Ideal Sperm Preparation Technique .....	19
1.5.3 Sperm Preparation Techniques .....	19
<b>1.6. Effect of insemination/injection time .....</b>	<b>21</b>
1.6.1 Reaching Nuclear Maturity.....	21
1.6.2 Incubation Time before IVF.....	21
1.6.3 Incubation time before ICSI .....	22
1.6.4 Oocyte Preincubation and Sperm Preparation.....	22
<b>1.7. DNA Damage.....</b>	<b>23</b>
1.7.1 DNA Structure .....	24
1.7.2 Aetiology of DNA damage.....	25
1.7.2.1 Chromatin packaging abnormalities.....	25
1.7.2.2 Abortive apoptosis .....	25
1.7.2.3 Oxidative stress .....	26
1.7.2.4 Cryopreservation .....	27
1.7.2.5 Extrinsic factors .....	28
1.7.3 Role of oocyte in sperm DNA damage.....	28
1.7.4 Assessment of Sperm DNA damage .....	28
1.7.4.1 DNA/Chromatin Integrity .....	29
1.7.4.2 Chromatin Packaging .....	31
1.7.5 Effect on ART Outcome .....	32
1.7.5.1 Fertilization .....	32
1.7.5.2. Embryo quality .....	32
1.7.5.3 Pregnancy.....	33
1.7.5.4 Offspring .....	34
<b>1.8 The research question .....</b>	<b>34</b>
<b>1.9. Aims and Objectives .....</b>	<b>35</b>
 <b>CHAPTER 2: MATERIALS AND METHODS .....</b>	 <b>36</b>
2.1 PRIMARY OBJECTIVE.....	36
2.1.1 Study Population.....	36
2.1.2 Sample Collection and Evaluation .....	36
2.1.3 Semen Evaluation, Preparation and Incubation.....	37

2.1.4 CMA <sub>3</sub> Procedure .....	41
2.1.5 TUNEL Procedure .....	42
2.1.6 Statistical Analysis .....	43
2.2 SECONDARY OBJECTIVE .....	44
2.2.1 Study Population.....	44
2.2.2 Ovarian Stimulation and Oocyte Collection.....	44
2.2.3 Semen Evaluation and Preparation .....	44
2.2.4 Fertilization Techniques .....	46
2.2.5 Timing .....	47
2.2.6 Assessment of Fertilization and Embryo Quality.....	47
2.2.7 Embryo Transfer and establishment of Pregnancy .....	48
2.2.8 Outcomes analyzed .....	49
2.2.9 Statistical analysis .....	49
<b>CHAPTER 3: RESULTS .....</b>	<b>50</b>
<b>3.1 Primary Objective 1:.....</b>	<b>50</b>
<b>The effect of incubation time and temperature on sperm motility and DNA of the non-processed semen sample.....</b>	<b>50</b>
3.1.1. Effect of Incubation Time and Temperature .....	52
3.1.1.1 <i>Motility – non-processed sample</i> .....	52
3.1.1.2. <i>CMA<sub>3</sub> – non-processed sample</i> .....	54
3.1.1.3. <i>TUNEL – non-processed sample</i> .....	55
3.1.2. Summary of Prospective Study 1 .....	57
<b>3.2. Primary Objective 2:.....</b>	<b>58</b>
<b>Study 2: The effect of incubation time and temperature on sperm motility and DNA of the processed semen sample .....</b>	<b>58</b>
3.2.1 Effect of Incubation Time and Temperature .....	60
3.2.1.1. <i>Motility – processed sample</i> .....	60
3.2.1.2 <i>CMA<sub>3</sub>– post-preparation</i> .....	62
3.2.1.3 <i>TUNEL– post-preparation</i> .....	63
3.2.1.4 <i>TUNEL and CMA3 correlation – post-preparation</i> .....	63
3.2.2. Summary: Prospective Study 2 .....	64
<b>3.3 Secondary objective: The effect of incubation time on the ART outcome – a retrospective study .....</b>	<b>65</b>
3.3.1 Effect of Sperm Incubation Time, Post-Preparation, on Fertilization Rates in IVF and ICSI .....	68



3.3.2 Effect of Sperm Incubation Time, Post Preparation, on Embryo Quality in IVF and ICSI .....	70
3.3.3 Effect of Sperm Incubation Time, Post-Preparation, on Pregnancy Rates in IVF and ICSI .....	72
3.3.4 Summary.....	75
<b>CHAPTER 4: DISCUSSION AND CONCLUSION.....</b>	<b>76</b>
<b>Bibliography .....</b>	<b>86</b>
<b>Addendum I: Semen Analysis estimation .....</b>	<b>106</b>
<b>Addendum II: D-Q Staining Method.....</b>	<b>109</b>
<b>Addendum III: Standard Swim-up Preparation .....</b>	<b>110</b>
<b>Addendum IV: SilSelect Gradient Preparation .....</b>	<b>111</b>
<b>Addendum V: TUNEL Method .....</b>	<b>113</b>
<b>Addendum VI: CMA<sub>3</sub> Method.....</b>	<b>116</b>
<b>Addendum VII: Oocyte Retrieval Protocol .....</b>	<b>117</b>
<b>Addendum VIII: IVF Procedure.....</b>	<b>118</b>
<b>Addendum IX: ICSI Procedure .....</b>	<b>119</b>
<b>Addendum X: Embryo grading day 2 and 3 .....</b>	<b>122</b>
<b>Addendum XI: Blastocyst Grading .....</b>	<b>123</b>

## LIST OF FIGURES

- Figure 2.1** Study 1: The incubation of non-processed semen samples at different times and temperatures
- Figure 2.2** Study 2: The incubation of processed semen samples at different times and temperatures
- Figure 2.3** A schematic representation of the timings of sperm incubation
- Figure 3.1** The LS means of the motility (%) after incubation of the non-processed semen sample (n=36) in 6 incubation groups ( $p < 0.05$ )
- Figure 3.2** The LS means of the CMA<sub>3</sub> values after incubation of the non-processed semen sample (n=36) in 6 incubation groups ( $p > 0.05$ )
- Figure 3.3** The LS means of the TUNEL values after incubation of the non-processed semen sample (n=21) in 6 incubation groups ( $p > 0.05$ )
- Figure 3.4** The LS means of the motility (%) after incubation of the processed semen sample (n=33) in 6 incubation groups ( $p < 0.05$ )
- Figure 3.5** The LS means of the CMA<sub>3</sub> values (%) after incubation of the processed semen sample (n=33) in 6 incubation groups ( $p > 0.05$ )
- Figure 3.6** The LS means of the TUNEL values (%) after incubation of the processed semen sample (n=20) in 6 incubation groups ( $p > 0.05$ )
- Figure 3.7** Box and Whisker plot depicting the significant different incubation times of processed semen samples in IVF (n=212) and ICSI (n=688) ( $p < 0.01$ )
- Figure 3.8** Box and Whisker plot depicting the significant difference in the fertilization rates (%) in IVF (n=212) and ICSI (n=688) cycles ( $p < 0.05$ )

**Figure 3.9** Lowess Smoother graph showing the significant negative association between incubation time and fertilization rates in IVF cycles (n=212) (p=0.041)

**Figure 3.10** Lowess Smoother graph showing the positive effect of incubation time on the embryo quality of day 2 embryos in IVF and ICSI cycles (n=904) (p=0.025)

**Figure 3.11** Box and Whisker plot depicting the significant different sperm incubation times between pregnant and non-pregnancy couples (p<0.05)

**Figure 3.12** Lowess Smoother plot showing the effect of sperm incubation time on pregnancy in IVF (n=292) and ICSI (n=688) cycles

## LIST OF TABLES

<b>Table I</b>	Median (upper-lower quartile range) values of the sperm parameters of the non-processed semen sample when incubated at different times and temperatures before and after incubation
<b>Table II</b>	Median (lower-upper quartile range) values of the sperm parameters of the non-processed semen sample in the different incubation groups before and after incubation
<b>Table III</b>	Post Hoc test displaying where the significance in motility (%) in the LS means (Figure 3.1) between the different incubation groups of the non-processed semen sample lie
<b>Table IV</b>	Median (upper-lower quartile range) values of the sperm parameters over different temperatures and times before and after the semen sample was processed and incubated
<b>Table V</b>	Median (upper-lower quartile range) values of the sperm parameters between the different incubation groups before and after the semen sample was processed and incubated
<b>Table VI</b>	Post hoc test displaying where the significant difference in motility (%) (Figure 3.4) between the different incubation groups of the processed semen sample lie
<b>Table VII</b>	Descriptive statistics of the IVF and ICSI outcomes. Mean $\pm$ SD values are displayed
<b>Table VIII</b>	The fertilization and pregnancy rates for the different time intervals of processed semen samples. Mean $\pm$ SD values are displayed
<b>Table IX</b>	Number and percentage of good quality embryos on day 2, day 3 and day 5

**Table X** Different outcomes between pregnant and non-pregnant cycles in IVF and ICSI. Mean $\pm$ SD values are displayed

## ABBREVIATIONS

<b>AB</b>	Aniline Blue
<b>AI</b>	Artificial Insemination
<b>AOT</b>	Acridine Orange Test
<b>ART</b>	Assisted Reproduction Technologies
<b>ATP</b>	Adenosine Triphosphate
<b>Ca<sup>2+</sup></b>	Calcium
<b>cAMP</b>	Cyclic adenosine monophosphate
<b>CMA<sub>3</sub></b>	Chromomycin A3
<b>COMET</b>	Single Gel Electrophoresis Assay
<b>DFI</b>	DNA Fragmentation Index
<b>DNA</b>	Deoxyribonucleic Acid
<b>D-Q</b>	Diff-quick
<b>E2</b>	Estrogen
<b>GIFT</b>	Gamete Intrafallopian Transfer
<b>hCG</b>	Human Chorionic Gonadotropin
<b>HCO<sub>3</sub><sup>-</sup></b>	Bicarbonate
<b>ICSI</b>	Intracytoplasmic Sperm Injection
<b>IMSI</b>	Intracytoplasmic Morphologically Selected Sperm Injection

<b>IUI</b>	Intra-uterine Insemination
<b>IVB</b>	In vitro bevrugting
<b>IVF</b>	In Vitro Fertilization
<b>MII</b>	Metaphase II
<b>OPU</b>	Ovum Pick-up
<b>PICSI</b>	Physiological Intracytoplasmic Sperm Injection
<b>ROS</b>	Reactive Oxygen Species
<b>RT</b>	Room Temperature
<b>SCSA</b>	Sperm Chromatin Structure Assay
<b>SD</b>	Standard Deviation
<b>Topo II</b>	Topoisomerase II
<b>TUNEL</b>	Terminal Deoxynucleotidyl Transferase-mediated Nick End Labelling
<b>WHO</b>	World Health Organisation
<b>ZIFT</b>	Zygote Intrafallopian Transfer
<b>ZP</b>	Zona Pellucida

## **CHAPTER 1: BACKGROUND AND LITERATURE REVIEW**

### **1.1. INTRODUCTION**

Assisted Reproduction Technologies [ART] in all its different forms are now common practice in IVF laboratories worldwide. An integral part of ART is the semen of the male partner. In all ART procedures - including intra-uterine insemination [IUI], gamete intrafallopian transfer [GIFT], zygote intrafallopian transfer [ZIFT], in vitro fertilization [IVF], intracytoplasmic sperm injection [ICSI], physiological intracytoplasmic sperm injection [PICSI] and intracytoplasmic morphologically selected sperm injection [IMSI] - the semen sample is handled, processed, prepared and manipulated before use in the fertilization process. The way the semen sample is handled can have potential positive or negative effects on ART outcome.

Two of the very basic, but important factors that may impact sperm cell quality and function post ejaculation and preparation, are time and temperature exposure.



## 1.2. HISTORY OF IVF

The history of IVF dates as far back as the 1600s and for more than a century IVF has seen ground-breaking studies, new techniques and ethical challenges.

### **Discovery of the sperm cell and ovum**

The first observation of human sperm cells dates from as early as 1677. Von Leeuwenhoek was the first to observe the sperm cell microscopically and published the first description in 1678 (Clarke, 2006). Hartsoeker thought that the sperm cell contained a miniature human being, what historians now refer to as homunculus (Cohen, 2007). This theory was overturned in the 1770s when Spallanzani examined semen from different types of animals and confirmed the presence of sperm cells. He also produced the first example of IVF, showing that in frogs, oocytes evolve to tadpoles when they come in contact with sperm cells from the frog (Clarke, 2006; Birkhead and Montgomerie, 2009). The discovery of mammalian oocytes was only reported in 1827 by Von Baer - von Baer was also responsible for cataloguing the early stages of embryonic development (Clarke, 2006; Cohen, 2007). In the early 19<sup>th</sup> century, Prevos and Damas concluded that sperm cells are found in the testes and they furthermore demonstrated that motile sperm cells were essential for fertilization of frog oocytes (Clarke, 2006). It wasn't until the 1870s that fusion of the sperm cell and ovum, in particular fusion of the nucleus was documented by Hertwig and Fol. Hertwig and Fol found that the sperm cell forms the male pronucleus and fuses with the female pronucleus in the cytoplasm of a starfish oocyte (Clarke, 2006; Birkhead and Montgomerie, 2009). In the same year, van Beneden described a reduction of the chromosome number in the gametes (Clarke, 2006). He also described meiosis, the process by which the number of chromosomes is reduced (Hamoir, 1992).

### **Artificial insemination [AI]**

In 1785, John Hunter recorded the first AI case in which he inseminated a female patient with her husbands' sperm cells (Mcdonell, 2010). The first published account of AI was in 1838 by

Dr Girault. In 1866, Sims published a book on sterility which included a chapter on AI (Clarke, 2006).

## **Cryopreservation**

Spallanzani was the first to investigate the effect of low temperature on sperm cells (Clarke, 2006). On 1949, Polge and Rowson discovered the properties of glycerol and the effectiveness of its use in cryopreserving sperm cells (Polge and Rowson, 1952). The first human birth of cryopreserved semen was reported in 1953 by Bunge and Sherman (Clarke, 2006; Bahadur, 2000) and the first cryopreserved embryo pregnancy was reported by Trounson and Mohr (1983), but this resulted in a still birth. Zeilmaker described the first live birth of twins in 1984 (Wennnerholm, 2000).

## **IVF**

In 1880, Walter Heape reported the first embryo transfer in rabbits and obtained full term offspring (Edwards, 1996). In 1935, Pincus and Enzmann raised the possibility that mammalian eggs can undergo normal development in vitro (Pincus and Enzmann, 1935). Austin and Chang discovered sperm capacitation in 1951 and in 1958 Austin and Bishop published the first evidence of the acrosome reaction (Clarke, 2006; De Cassia da Silveira, 2010). The first IVF birth using capacitated sperm was achieved in rabbits in 1963 (Yanagimachi and Chang, 1976). Studies on oocyte maturation on several mammalian species were carried out in the late 1950s and early 1960s. The release of the human oocyte from the follicle, to germinal vesicle breakdown, 1<sup>st</sup> polar body excursion and entry in to metaphase II [MII] was described by Edwards in 1965 (Edwards, 1996). The first report on human IVF was published by Edwards in 1969. He used ejaculated spermatozoa that have been washed free of seminal plasma to achieve fertilization and went on to report embryonic development to the 16 cell stage (Edwards, 1996). In 1975, Edwards and Steptoe had a pregnancy, but it turned out to be ectopic. The first IVF birth, Louise Brown, occurred in England on the 25<sup>th</sup> of July 1978.

Other methods started to arise to treat infertility such as human preimplantation embryo biopsy, sexing by DNA amplification (Handyside *et al.*, 1989; Franklin and Roberts, 2006) and assisted hatching (Cohen *et al.*, 1990).

ICSI, injecting a sperm cell into the oocyte through micromanipulation, gave way to promising possibilities in treating almost all types of male infertility problems. The first baby born from ICSI was in 1992 (Palermo *et al.*, 1992).

### **1.3. MALE INFERTILITY AND STANDARD SEMEN PARAMETERS**

Infertility affects approximately 15% of couples trying to conceive and a male cause is responsible for about half of these cases (Oehninger, 2001). The cause of male infertility can be the result of congenital and acquired urogenital abnormalities, infections of the genital tract, varicocele, endocrine disturbance and genetic or immunological factors (WHO, 2010).

The basic diagnosis of male infertility relies on the conventional semen analysis. The semen analysis is a critical component of the initial workup of male infertility and can offer insight into the cause of infertility. The parameters usually assessed by semen analysis include ejaculate volume, sperm concentration, sperm motility and sperm morphology. Semen pH, viscosity, white blood cell concentration and the degree of sperm agglutination may also be reported. Other functional parameters that are assessed include sperm viability and antibody positive sperm evaluation.

Since 1980, the World Health Organisation [WHO] has published reference values for human semen parameters. These values have changed with the latest updated version from 2010 (WHO, 2010). The traditional semen analysis has been criticized, due to a lack of power in predicting fertility (Lewis, 2007). This may be due to the WHO analysis only taking sperm characteristics into consideration and not the sperm nuclear potential i.e. the sperm DNA. Even though traditional sperm cell characteristics are seen as poor fertility markers, it is the most important factor for male prognosis and choosing a suitable ART treatment.

#### **1.3.1 Semen Physiology**

Semen consists of 2 components: sperm cells and seminal plasma. Sperm cells are formed within the seminiferous tubules of the testes during spermatogenesis and constitute approximately 10% of the semen volume. The prostate, seminal vesicle and bulbourethral glands produce the seminal plasma, which makes up 90% of the semen volume.

### **1.3.1.1 Spermatogenesis**

The most immature male germinal cell is the spermatogonium. Spermatogonia first undergo mitotic divisions and proceed into spermatogenesis or degenerate. Type A spermatogonia, responsible for fertility preservation, differentiate into intermediate type B spermatogonia, which contain more chromatin. The type B spermatogonia are the immediate precursors of primary spermatocytes (Hoogendijk and Henkel, 2007) and after the first meiotic division they form two haploid secondary spermatocytes. The secondary spermatocytes go through the second meiotic division and produce two round haploid spermatids which carry half the DNA of secondary spermatocytes (Menéndez and Marina, 1999). Any anomalies in spermatogenesis may contribute to male infertility (Jégou *et al.*, 2002).

### **1.3.1.2 Spermiogenesis**

Spermiogenesis is the maturational process by which a haploid spermatid differentiates into a mature spermatid i.e. testicular spermatozoa and occurs once meiosis is completed. Nuclear histones are replaced by protamines (Kazerooni *et al.*, 2009), chromatin condenses and the compacted nucleus assumes a position close to the cell membrane. The Golgi complex produces the acrosome, which is applied close to the nucleus in contact with the cell membrane. One of the centrioles attaches to the pole of the nucleus opposite the developing acrosome and produces the axial filament. Cytoplasmic reduction of the spermatid occurs and the mitochondria are arranged around the proximal part of the axial filament complex of the developing tail.

Spermiation follows, which is where residual cytoplasm is shed from the neck region of the spermatid and the spermatid is released from the seminiferous epithelium into the tubule lumen.

### **1.3.1.3 Post-testicular sperm maturation**

Post-testicular sperm maturation occurs in the epididymis and involves a combination of morphological, biochemical, biophysical and metabolic changes. The sperm cells acquire motility and become capable of fertilizing, dependent on the final maturational process of capacitation. The surface of the sperm cell changes and the final chromatin condensation and stabilization occurs.

If the sperm cells are stored in the epididymis for too long, the sperm cells lose their fertilizing ability, motility and viability. The senescent sperm cells must be eliminated from the male tract at regular intervals; otherwise the semen quality will decrease (section 1.4.3).

### **1.3.1.4 Seminal Plasma**

Seminal plasma is mainly produced from the prostate, seminal vesicles and the bulbourethral glands.

Sperm are expelled in the first ejaculate portions together with the zinc-rich secretion from the prostate gland (Bjorndahl, 2010). The prostate secretion does not coagulate spontaneously upon emission and contains fibrinolytic enzymes responsible for liquefaction of the coagulated vesicular secretion. It also acts to enhance sperm motility. The seminal vesicles produce the vesicular fluid which contributes 70% of the semen volume. The vesicular fluid is expelled after the prostatic secretion and contains much lesser sperm (Bjorndahl, 2010). Fructose is one of the main components of the vesicular fluid and other constituents include ascorbic acid, inorganic phosphorus, potassium and prostaglandins.

The bulbourethral glands secrete a clear and mucoid fluid which neutralizes acidic urinary residue in the urethra before ejaculation of the sperm-rich first fraction of the ejaculate.

### 1.3.2 Significance of Semen Parameters

Semen parameters (sperm concentration, morphology and motility) are a reflection of testicular function and to a lesser extent, post-testicular genital tract function (Zini and Libman, 2006). There is little consensus as to which parameter is the best predictor of fertility.

#### 1.3.2.1 Sperm Motility

Sperm motility is an important functional characteristic and a prerequisite for fertilization. Sperm motility is time and temperature dependent and assessment should be carried out as close to body temperature as possible. Prolonged incubation at 37°C may reduce sperm motility (Calamera *et al.*, 2001; Lachaud *et al.*, 2004) (section 1.4.4). There are conflicting reports on the effect of sperm motility on fertilization and pregnancy rates (Sukcharoen and Keith, 1996). Shulman *et al.* (1998) stated that the only parameter that could predict the treatment outcome was the percentage of motile sperm cells after sperm preparation.

#### 1.3.2.2 Sperm Concentration

The number of sperm cells in the ejaculate is also of paramount importance. Although sperm concentration is associated with the likelihood of achieving a pregnancy, there are no absolute limits to distinguish fertile from infertile except azoospermia (Mortimer and Mortimer, 2005). Azoospermia is the absence of sperm cells in the ejaculate and may occur from ejaculatory dysfunction, obstruction of the reproductive tract, or as a result of abnormal sperm cell production (WHO, 2010). Bonde *et al.* (1998) found that the probability of conception increased with increasing sperm concentration up to 40x10<sup>6</sup>/ml. When the sperm concentration was higher than 40x10<sup>6</sup>/ml, the probability of conception did not increase.

### **1.3.2.3 Sperm Morphology**

Sperm morphology has been reported to be the most significant determining factor in predicting the outcome of an IVF cycle (Kruger *et al.*, 1986; Liu and Baker, 1992). The use of strict criteria (Menkveld *et al.*, 1990) for the evaluation of morphology is an important factor in predicting fertilization and pregnancy rates. The probability of achieving fertilization increases with an increase in the morphology percentage. A morphology reading below 4% decreases the chance of fertilization. Sperm morphology also correlates significantly with sperm motility and its ability to bind to the zona pellucida [ZP] (Menkveld *et al.*, 1991; Liu and Baker, 1992). Furthermore, there is a strong correlation between normal sperm morphology and the induction of the acrosome reaction (Menkveld *et al.*, 1996).

A study comparing men with proven fertility with men undergoing infertility investigations concluded that sperm concentration and motility were better predictors of fertility potential than sperm morphology as assessed by WHO guidelines and Tygerberg strict criteria (Nallella *et al.*, 2006).



### 1.3.3 Maturation of the sperm cells

Upon ejaculation, sperm cells are motile but unable to undergo fertilization and only become competent as they travel through the different environments in the female reproductive tract. The sperm cells undergo biochemical and physiological changes termed capacitation. Hyperactivation of the sperm cell is initiated and they are able to bind to the ZP of the oocyte. Once the sperm cell binds to the oocyte the acrosome reaction can occur and sperm-oocyte fusion can take place.

#### 1.3.3.1 Capacitation

The first step in sperm activation is capacitation, a gradual process where sperm cells undergo biochemical and physiological changes that are required for hyperactivation and the acrosome reaction. Capacitation acts to remove seminal plasma proteins attached to the sperm cells and it modifies and reorganizes sperm plasma membrane molecules (Abou-haila and Tulsiani, 2009). Events that occur during capacitation include cholesterol efflux from the sperm plasma membrane, increased membrane fluidity, modulation in intracellular ion concentration, hyperpolarization of the ZP and increased protein phosphorylation. Capacitation must occur at a controlled rate so that sperm cells can reach the oocyte at the appropriate physiological state (Abou-haila and Tulsiani, 2009). The endpoint of capacitation is to achieve hyperactivity so that the sperm cell can bind to the intact ZP of the oocyte and release its acrosomal contents.

The process of capacitation in vivo commences after ejaculation, where the sperm cells are exposed to different environments in the female reproductive tract. As the sperm cells move along the tract, capacitation is initiated and subsequently changes to the plasma membrane of the sperm cell occurs. Cholesterol is a very important sterol in the plasma membrane as it regulates the fluidity of the membrane during capacitation and the acrosome reaction. When capacitation is initiated, there is an efflux in cholesterol that changes permeability and allows an influx of Calcium [ $\text{Ca}^{2+}$ ] and bicarbonate [ $\text{HCO}_3^-$ ] ions, which in turn activates intracellular

secondary messengers (Abou-haila and Tulsiani, 2009). This efflux of cholesterol is also critical for the activation of protein kinases which act to increase tyrosine phosphorylation, leading to a change in the protein conformation of the sperm plasma membrane (Visconti and Kopf, 1998; De Cassia Dá Silveira e Sa, 2010). Cholesterol efflux therefore controls the balance between protein phosphorylation and dephosphorylation (Osheroff *et al.*, 1999; Visconti *et al.*, 1999). The flagellum is a major component of sperm cells that undergo tyrosine phosphorylation and is related to the acquisition of hyperactivation. All these modifications are crucial for the acrosome reaction to occur.

The distribution pattern of cholesterol in the sperm membrane is also changed with capacitation: in non-capacitated sperm cells it is found over the entire sperm cells' head and after the acrosome reaction is induced, it is removed from the equatorial to the apical region of the sperm head (Pasqualotto *et al.*, 2010; Sutovsky, 2010).

Reactive oxygen species [ROS] play an important role in capacitation and acrosome reaction by initiating and controlling the  $\text{Ca}^{2+}$  influx, intracellular pH and cAMP rise, increased membrane fluidity, activation of signal transduction cascades and protein phosphorylation (De Cassia Dá Silveira, 2010). Bicarbonate is also important in activating protein kinases and changing the membrane fluidity (De Cassia Dá Silveira, 2010; Ickowicz *et al.*, 2012).

### **1.3.3.2 Hyperactivation**

Hyperactivation is important for fertilization as it facilitates the sperm cells as it progresses up the oviduct and helps to penetrate the ZP. An influx of  $\text{HCO}_3^-$  is regulated by  $\text{Ca}^{2+}$  activity and is a major determinant in the development of hyperactivated motility (De Cassia Dá Silveira, 2010).

#### **1.3.3.3 The acrosome reaction**

The sperm cells' acrosome is a Golgi-derived secretory granule that is formed during early stages of spermatogenesis (section 1.3.1.2). It is a sac-like structure surrounded by inner- and outer acrosomal membranes. The acrosome reaction is an exocytotic process induced by contact with ZP receptors (ZP3) on the oocyte, and leads to fusion of the outer and inner acrosomal membranes. Only capacitated sperm can undergo the acrosome reaction. The contents of the acrosome (acrosin and hyaluronidase) are released and these enzymes help the sperm cells go through the cumulus, corona radiata and ZP (Pasqualotto *et al.*, 2010). The acrosome-reacted sperm cells will not be able to bind to the ZP of the oocyte due to missing sperm receptors that are present on the acrosome-intact sperm cells (de Cassia Dá Silveira, 2010). Acrosome-reacted sperm cells can then go through the ZP and into the perivitelline space. Once inside the perivitelline space, the penetrating sperm cell fuses with the oocyte (Pasqualotto *et al.*, 2010). Sperm fusion is temperature, pH and  $\text{Ca}^{2+}$  dependent (Pasqualotto *et al.*, 2010).

#### **1.3.3.4 Capacitation and the acrosome reaction in vitro**

Capacitation in vitro is initiated by incubating the sperm cells in a chemically defined medium supplemented with energy substances such as pyruvate, lactate, glucose, a cholesterol acceptor (usually serum albumin),  $\text{NaHCO}_3^-$ ,  $\text{Ca}^{2+}$ , low  $\text{K}^+$  and physiological  $\text{Na}^+$  concentrations (Visconti *et al.*, 1995; De Cassia Dá Silveira, 2010). Serum albumin is an essential component of in vitro capacitation media, as it decreases cholesterol by removing it from the sperm plasma membrane. This effect is dependent on the presence of  $\text{HCO}_3^-$  (Cross, 1998). Capacitation is inhibited by the addition of cholesterol and or cholesterol analogues to the capacitation medium (Visconti *et al.*, 1999). Tyrosine phosphorylation of proteins also plays an important role in capacitation and it is dependent on the presence of serum albumin,  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  in the medium (Visconti *et al.*, 1995).

After incubation in defined media, the capacitated sperm cells need to penetrate the cumulus oophorus and bind to the ZP to trigger the acrosome reaction. This is done in vitro by preparing the sperm suspension through a discontinuous density gradient or by washing and centrifuging the sample. Afterwards the sperm cells are incubated in  $\text{HCO}_3^-$  enriched media (Abou-haila and Tulsani, 2009). When the sperm cells are incubated with the oocytes in vitro, the cumulus is rapidly colonized by the washed sperm and capacitation-related changes in the plasma membrane of the sperm cell helps the sperm to bind to the ZP (Pasqualotto *et al.*, 2010).

Temperature changes in the sample may affect processes in capacitation that can affect the membrane lipid diffusibility and peroxidation, as well as antigen distribution on the sperm cell plasma membrane (Villarroya and Scholler, 1987). This can cause changes in ion permeability (especially  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$ ) and in the activity of the membrane-bound enzymes (Harrison and Gadella, 1995). Temperature changes in vitro may also affect capacitation due to phosphorylation on tyrosine residues. Marin-Briggiler *et al.* (2002) found that capacitation is inhibited at 20°C and speculated that a temperature of 20°C blocks the removal of cholesterol and subsequently inhibits protein tyrosine phosphorylation (Marin-Briggiler *et al.*, 2002).

## **1.4. SEMEN HANDLING AND INCUBATION**

### **1.4.1 Production Method**

Masturbation is the preferred method of production and should be performed in a room close to the laboratory. Producing a sample by masturbation under the controlled conditions of a dedicated room on site provides control of the environmental conditions, reduces the time between collection and analysis and the technician is able to observe coagulation and liquefaction.

### **1.4.2 Container**

A 60-100ml wide-mouthed sterile polypropylene jar with a screw cap is the most popular and recommended type of container for semen collection. Other options include a special condom or coitus interruptus, but these methods are not recommended.

### **1.4.3 Days of Abstinence**

An abstinence of 2-5 days has been recommended and this primarily helps to standardize the conditions and reduces sample variation (Jurema et al., 2005). The abstinence period is also important to ascertain if the correct type of sample is evaluated, as prolonged abstinence influences the volume and motility of the sperm cells.

### **1.4.4 Exposure Time and Storage Temperature**

Between masturbation and in vitro fertilization, the semen sample is first incubated to allow liquefaction and await preparation and thereafter it is incubated to await the appropriate ART procedure. The time intervals and temperatures at which these samples are prepared and stored vary greatly due to numerous factors. The WHO does not recommend a specific temperature at which these procedures should be performed. A temperature range from 20°C - 37°C is mentioned (WHO, 2010).

#### **1.4.4.1 First Incubation: Semen Collection to Sperm Preparation**

The WHO recommends that the male should produce the semen sample in a private room near the laboratory (section 1.4.1). However, upon request of the patient, the samples are often brought in from home. Patients are advised that semen samples produced at home should be delivered to the laboratory within one hour of collection and the sample should be protected from temperature extremes during transport (WHO, 2010). The ejaculated semen sample is usually incubated at 37°C until preparation begins (Van der Westerlanken *et al.*, 2006).

Time (Appell and Evans, 1977; Appell *et al.*, 1977) and temperature (Esfandiari *et al.*, 2002) have a negative effect on sperm progressive motility in fresh (unwashed) semen samples. However, studies have shown that, in a fresh semen sample, sperm cells can preserve their motility for up to 12 hours (Appell *et al.*, 1977). Therefore, the 1 hour time-span recommendation by the WHO is suitable for diagnostic semen analysis. This recommendation is however not suitable for therapeutic semen processing for ART procedures. Yavas and Selub (2004) found that samples incubated for 15-30 minutes (37°C) prior to preparation resulted in higher pregnancy rates in IUI patients compared to samples that were incubated for 30-60 minutes. No pregnancies were found when the time interval from semen collection to preparation exceeded 60 minutes.

After the sperm cell is ejaculated, it is exposed to seminal plasma. The seminal plasma configures the extracellular milieu and its contents may play important roles in capacitation. Seminal vesicle fluid is a major component of seminal plasma and contains many factors such as activators of sperm motility, energy sources (fructose and citric acid), prostaglandins that suppress the female immune response and a semen coagulation factor that suppresses sperm metabolism (section.1.3.1.4) The seminal plasma contains macromolecules (cholesterol) that are able to decapacitate already capacitated sperm. Decapacitation factors usually prevent premature capacitation (Begley and Quinn, 1982), but over-exposing the sperm cells to these

factors irreversibly prevents capacitation, the acrosome reaction and in turn impairs the sperm cells' fertilizing ability (Yavas and Selub, 2004).

Seminal plasma also contains leukocytes and dead sperm cells that produce ROS, which at high levels can have adverse effects on these cells. ROS are important in the control of normal sperm function (Aitken, 1995; Agarwal *et al.*, 2003), but exposing the sperm cells for too long to ROS leads to lipid peroxidation (impairing the sperm cell membrane) and loss of motility and viability of the sperm cells (Agarwal and Said, 2003). ROS can also induce premature capacitation (Villegas *et al.*, 2003). Hence, sperm cells must be separated from the seminal plasma as soon as possible after ejaculation (section 1.5.1).

#### **1.4.4.2 Second Incubation: Sperm Preparation to ART procedure**

After the sample is processed, it is usually incubated at 37°C for at least 1-2 hours before injection or insemination. The incubation of sperm cells is important for the acrosome reaction to occur. However, prolonged incubation can have a negative effect on the sperm fertilizing ability. There is no determined agreement on optimal time for incubation of sperm cells prior to ART. A recent study did recommend a 40-80 minutes sperm incubation period after sperm preparation and before IUI. The temperature at which the sperm sample should be incubated was not specified (Faque *et al.*, 2014). Sperm motility requires energy sources such as glucose and fructose (Williams and Ford, 2001). If the sources are exhausted, the sperm cells won't be able to reach the fertilization site. Premature capacitation can lead to the acrosome reaction occurring early. In both IVF and ICSI, the medium in which the sperm cells are incubated plays an important role in initiating the acrosome reaction and should be kept at 37°C (Schill *et al.*, 1988).

It has been shown that incubation of sperm at 37°C for more than 4 hours increases sperm DNA fragmentation (Dallzell *et al.*, 2004). Additionally, there is an increase in sperm cells with uncondensed chromatin with in vitro incubation at 37°C during a 24 hour time span (Hammadeh *et al.*, 2001). A recent study by Faque *et al.* (2014) recommends a 40-80 minutes

sperm incubation period after the sample is processed and before IUI. The temperature at which the sperm sample should be incubated was not specified.



## 1.5. SPERM PREPARATION

Prior to starting a treatment cycle, semen parameters are assessed and from the assessment, a method of sperm preparation is recommended. The aim of sperm preparation is to remove the seminal plasma components and maximize the yield of normal, high motility sperm cell fraction.

### 1.5.1 Removal of the seminal plasma

The coagulating and liquefying processes of seminal plasma help sperm cells penetrate the cervical mucus but some of its components are obstacles to the achievement of pregnancy when natural barriers are bypassed in ART. Semen preparation involves removal of the seminal plasma (section 1.2.1.4 and 1.4.4.1), which contains senescent sperm cells, leukocytes, epithelial cells, debris and microbial contamination. Seminal plasma also contains factors that inhibit the fertilizing ability of the sperm cells and reduce the induction of capacitation (Mortimer, 2000). Leukocytes or abnormal sperm cells release ROS and affect the normal sperm cells in the sample. ROS production can be avoided by 1] antioxidants naturally present in the seminal plasma, 2] the protein supplement in the medium, 3] minimizing centrifugation and 4] separating the sperm cells from the leukocytes, dead sperm cells and debris early in the preparation procedure while seminal plasma is still present (Flint *et al.*, 2012).

Through the removal of the sperm cells from seminal plasma and the exposure to protein-containing buffers, capacitation of the sperm cells occur. As mentioned, the sample is incubated in a defined medium that is necessary for capacitation (section 1.3.3.4). Capacitation is an important prerequisite for the acrosome reaction and subsequently fertilization.

### 1.5.2 Ideal Sperm Preparation Technique

The ideal sperm preparation method should minimize damage to the sperm cells. Dilution, temperature change, centrifugation and exposure to potentially toxic material must be considered. Dilution should be performed slowly. The media suggested for sperm preparation is a balanced salt solution that contains moderate to high concentrations of protein and an appropriate buffer (Aitken *et al.*, 1989; WHO, 2010). Depending on the incubator, HEPES or bicarbonate buffers are used to ensure that the culture pH is compatible with sperm survival (WHO, 2010). Temperature changes should be gradual and sperm preparation procedures should be performed at 37°C. The lowest possible force should be used for centrifugation and centrifugation should be kept to a minimum. Furthermore, the sperm preparation method should aim to be quick and cost-effective, isolate as many motile sperm cells as possible, eliminate dead sperm cells and other cells, eliminate toxic or bioactive substances and allow processing of large volumes of ejaculates (Henkel and Schill, 2003).

Sperm preparation is also incorporated in semen cryopreservation protocols. Semen cryopreservation results in a decrease in motility (section 1.7.2.4) and must therefore be processed in order to enrich the specimen with higher quality sperm cells (Sharma and Argawal *et al.*, 1996).

### 1.5.3 Sperm Preparation Techniques

With the introduction of ART, a range of sperm separation methods were developed that can be differentiated in migration, density gradient centrifugation and filtration techniques. These techniques are effective in selecting the most motile, morphologically normal sperm cells. The choice of technique is dictated by the nature of the semen sample.

The swim-up technique can be used for more normozoospermic samples, whereas the density gradient centrifugation technique is preferred in cases of severe oligozoospermia, teratozoospermia or asthenozoospermia. The density-gradient technique results in a higher

sperm concentration and a higher progressive motile sperm cell recovery rate, while the swim-up results in better motility (Boomsma *et al.*, 2004).

Sperm preparation techniques involving centrifugation have been shown to have an impact on DNA integrity. However, studies have shown that the levels of DNA fragmentation decrease after preparation (Gandini *et al.*, 2004) and the preparation technique seems to have an effect on the levels of fragmentation (Zini *et al.*, 2000). Swim-up and density gradient centrifugation have been shown to be efficient in decreasing sperm DNA fragmentation and improve DNA integrity (Zini *et al.*, 2000; Gandini *et al.*, 2004).

## 1.6. EFFECT OF INSEMINATION/INJECTION TIME

After oocyte retrieval, the oocytes are incubated to await insemination or injection with either IVF or ICSI. The time between oocyte retrieval and insemination/injection varies. Studies are contradictory to the effect of incubation time on fertilization and pregnancy rates (Khan *et al.*, 1989; Van de Velde *et al.*, 1998; Yanagida *et al.*, 1998).

### 1.6.1 Reaching Nuclear Maturity

Oocytes are considered to be meiotically mature (MII) after extrusion of the first polar body. However, nuclear and cytoplasmic maturity are acquired independently during oocyte maturation (Rienzi *et al.*, 1998). Cytoplasmic maturity is thought to be asynchronous with nuclear maturity (Eppig *et al.*, 1994) and incubation of the oocytes may induce cytoplasmic maturation and subsequently nuclear maturity. Evidence suggests that the maturity of the oocytes affect the outcome of IVF in both the fertilization rate and embryo quality (Zenzes *et al.*, 1985). It has been reported that a preincubation period can decrease the premature condensation rate of immature oocytes (Calafell *et al.*, 1991). On the other hand, prolonged culture of oocytes can lead to zona hardening, increased parthenogenetic activation and impaired embryonic development (Fukuda *et al.*, 1992).

### 1.6.2 Incubation Time before IVF

It has been shown that a delay of 2-6 hours prior to IVF improves fertilization and pregnancy rates (Trounson *et al.*, 1982; Khan *et al.*, 1989). A recent study found that a preincubation period of less than 2.5 hours leads to decreased fertilization rates and suggests that delaying insemination for 2.5-5.5 hours is beneficial to fertilization rates (Ho *et al.*, 2003).

### 1.6.3 Incubation time before ICSI

The optimal time for ICSI has not been established and studies are contradictory (Patrat *et al.*, 2012). Ho *et al.* (2003) found that preincubation of oocytes for more than 2.5 hours is beneficial for nuclear maturation in ICSI cycles.

Another factor which also influences the collection time of sperm and duration of oocyte incubation before ICSI is the presence of a meiotic spindle in the oocyte. Oocytes with visible meiotic spindles have higher fertilization rates and better embryo development (Wang *et al.*, 2002). It has been shown that the meiotic spindle changes over time, and there is a specific time period where a maximum of spindles are detected and a time period where they start to disintegrate. Kilani *et al.* (2010) found that directly after oocyte retrieval (36-36.5h post hCG), 58% of oocytes had a meiotic spindle and this increased significantly to 96% over a time period of 4 hours (39-40.5 hours post hCG). Temperature fluctuations during oocyte retrieval may have an influence on the spindle and the stabilization of the temperature after retrieval leads to an increase in the presence of meiotic spindles. It is therefore recommended to inject the oocyte with ICSI 4 hours after oocyte retrieval (39-40.5 hours post hCG).

### 1.6.4 Oocyte Preincubation and Sperm Preparation

It is also important to consider the oocyte insemination/injection time to determine the best time for collecting and preparing the semen sample and to determine how the prepared sample should be stored (section 1.4.4). After ICSI, the sperm cell makes two important contributions to the oocyte: it contributes paternal DNA and it is the trigger that activates the oocyte to complete second meiotic division (Rienzi *et al.*, 1998). The sperm cell releases a factor that promotes oocyte activation. This factor appears to be heat-sensitive (Dozortev *et al.*, 1995). Failure of the sperm cell to release this factor may lead to fertilization failure.

## 1.7. DNA DAMAGE

As mentioned before, the conventional semen analysis is now recognized to be of limited value in the determination of a couple's fertility status (Lewis, 2007). Approximately 15% of patients with male factor infertility have a normal semen analysis and no diagnosis can be made (Agarwal and Allamaneni, 2005).

The introduction of ICSI has further reduced the diagnostic significance of parameters such as sperm concentration, motility and morphology. ICSI allows patients with male factor infertility to become genetic fathers by circumventing most of the biological processes involved in natural conception. The selection of the sperm cell to be used for ICSI is based on the judgement of the embryologist, who chooses a motile sperm cell with as good morphologic appearance as possible. However, the selected sperm cell may have damaged DNA. Infants conceived by ICSI have been reported as being at an increased risk of malformations (Nasr-Esfahani *et al.*, 2005).

Sperm DNA testing has been increasingly regarded as a more promising test for fertility prediction. Sperm cells of infertile men contain more DNA damage than that of fertile men and this damage has a negative impact on the fertility potential of a couple. It has also been shown that 8% of men with normal semen parameters have sperm DNA damage (Zini *et al.*, 2001). There appears to be a threshold of sperm DNA damage beyond which embryo development and pregnancy are impaired (Ahmadi and Ng, 1999; Cho *et al.*, 2003). Fertilization by sperm cells containing fragmented DNA may lead to fetal mutations and may also increase the risk of cancer in the offspring. (Hansen *et al.*, 2002; Aitken and Baker, 2006). Furthermore, fertilization by sperm cells with fragmented DNA results in poor embryonic development, decreased implantation, lower pregnancy rates and recurrent pregnancy losses (Chohan *et al.*, 2006).

### 1.7.1 DNA Structure

Mammalian sperm cells are the cells with the most tightly compacted eukaryotic DNA (Ward and Coffey, 1991). During spermatogenesis (section 1.2.1 and 1.2.2) the spermatid nucleus is remodelled and condensed, which is associated with the displacement of histones by transition proteins and then by protamines (Steger *et al.*, 2000). DNA strands are tightly looped around the protamine molecules and inter- and intramolecular disulfide cross-links between the cysteine-rich protamines are responsible for the compaction and stabilisation of the sperm nucleus. This nuclear compaction acts to protect the sperm genome from external stresses and permits transfer of the very tightly packed genetic information to the oocyte (Zini and Libman, 2006). In addition to these protective measures, sperm cells are immersed in fluids containing huge levels of antioxidants (Singh *et al.*, 2003).

Up to 15% of the sperm cells' chromatin is packaged less tightly by histones at specific DNA sequences. It is thought that these DNA sequences are involved in fertilization and early embryo development (Wykes and Krawetz, 2003). It has been shown that infertile men have an increased sperm cell histone protamine ratio (Steger *et al.*, 2000; Oliva, 2006). An increase of histones results in poorer chromatin compaction and increased sensitivity to external stresses (Kosower *et al.*, 1992). A small portion of sperm cell DNA is of mitochondrial origin which exhibits a high rate of mutation and is related to the motility of the sperm cell (Kao *et al.*, 1998).

Instead of exhibiting a homogeneous chromatin structure, a considerable proportion of sperm cells have a coarse, granular appearance with vacuoles (Jager, 1990). This coarse chromatin may be indicative of immaturity due to inadequate time in the epididymis (Seligman *et al.*, 1994).

### **1.7.2 Aetiology of DNA damage**

The aetiology of sperm DNA damage is multi-factorial and may be due to intrinsic or extrinsic factors. Abnormal sperm cell chromatin/DNA structure is thought to arise from three possible sources: 1) chromatin packaging abnormalities; 2) abortive apoptosis; and 3) oxidative stress (Sakkas *et al.*, 1999; Agarwal *et al.*, 2003).

#### **1.7.2.1 Chromatin packaging abnormalities**

During spermiogenesis, sperm cell chromatin undergoes an important step in remodelling in which histones are replaced by protamines. This chromatin remodelling is facilitated by the coordinated loosening of chromatin by histone hyper-acetylation and the enzyme DNA topoisomerase II [Topo II]. This produces temporary nicks in the sperm DNA to relieve torsional stress resulting from supercoiling (Marcon and Boissonneault, 2004). These temporary nicks are then normally replaced by the same enzyme, Topo II, prior to completion of spermiogenesis and ejaculation. However, if these nicks are not repaired, DNA fragmented sperm cells may be present in the ejaculate (Muratori *et al.*, 2006). An increased sperm cell histone: protamine ratio is found in infertile men. This increase induces DNA damage and may occur due to a defect in spermatogenesis.

#### **1.7.2.2 Abortive apoptosis**

DNA breakage could be a consequence of an apoptotic like DNA degradation process, although this theory has been challenged (Sakkas *et al.*, 2003; Muratori *et al.*, 2000). Apoptosis is a process of programmed cell death that occurs in many cells throughout the body. In the testes, apoptosis normally occurs to prevent the overproduction of germ cells and to selectively destroy injured germ cells. Sertoli cells are only able to support a limited amount of germ cells. Thus when the germ cells are in excess, apoptosis is necessary to limit the germ cell population (Sinha and Swedloffn, 1999). It has been suggested that an early apoptotic



pathway, initiated in spermatogonia and spermatocytes, is mediated by Fas protein, which is secreted by Sertoli cells (Lee *et al.*, 1997). Men with poor seminal parameters often display a large percentage of Fas expressing sperm cells in the ejaculate. Some of these sperm cells with DNA damage and Fas expression have undergone “abortive apoptosis”, in which they started, but escaped the apoptotic pathway (Sakkas *et al.*, 1999). The use of Annexin V – conjugated microbeads is a selection method that can distinguish the non-apoptotic from the apoptotic sperm cells.

### **1.7.2.3 Oxidative stress**

Oxidative stress has long been implicated as the major etiological factor in sperm cell DNA damage. A low physiological level of ROS is accepted as necessary to maintain normal sperm function (Agarwal *et al.*, 2003), but if ROS levels exceed physiological norms they lead to deteriorating function or reduced survival (Aitken and Baker, 2002). Increased levels of ROS have been reported in semen of approximately 40% of infertile men (Zini *et al.*, 1993). Additionally, a positive correlation was found between sperm DNA fragmentation and ROS (Barroso *et al.*, 2000).

Major sources of ROS in semen are leukocytes and the sperm cells themselves, especially the immature sperm cells that still carry residual cytoplasm (Schulte *et al.*, 2010). In oligozoospermic samples, the sperm cells themselves are the major source of excess ROS generation (Aitken *et al.*, 1992). Leukocytes, even at low concentrations, are associated with abnormal sperm cell function due to their high ROS generation (Aitken *et al.*, 1995).

Centrifugal pelleting of sperm cells causes the production of ROS in the pellet and leads to irreversible damage to the sperm cell (Aitken and Clarkson, 1988) and sperm DNA (Muratori *et al.*, 2003) (section 1.4.2). This impairs the fertilizing potential of the motile sperm cell fractions (Mortimer, 2000). This indicates that when seminal plasma is removed, leukocyte-

derived or sperm-derived ROS exposure can induce apoptotic or necrotic events and lead to impaired fertilization potential (Evenson and Wixon, 2006). ROS production may be enhanced 20- to 50 fold during repeated centrifugation (Aitken and Clarkson, 1988).

#### **1.7.2.4 Cryopreservation**

Cryopreservation is widely used to preserve male gametes and provide the opportunity for future fertility. Sperm cells seem to be less sensitive to cryopreservation damage because of high fluidity of the membrane and low water content. However, freezing may lead to deleterious changes of sperm cell structure and function (Watson, 2000). The most commonly reported detrimental effect is a marked reduction in motility which decreases by approximately 30% in both fertile and infertile patients (Cathcart *et al.*, 1984). Liquid phase transition changes and increased lipid peroxidation lead to irreversible rupturing of plasma and nuclear membranes and disturbance of cellular organelles (Donnelly *et al.*, 2001). Studies suggest that the nucleus does not remain stable and that inappropriate chromatin condensation can occur (Royere *et al.*, 1991). There is no agreement in the literature on whether cryopreservation induces DNA damage (Di Santo *et al.*, 2012). Some studies show that freeze-thawing of sperm cells results in a reduction of the sperm cell metabolism which reduces the number of functional sperm cells (Hammadeh *et al.*, 1999; Spano *et al.*, 1999; de Paula *et al.*, 2006). The mechanism by which DNA damage is induced is unclear. Other studies show that fertile men are less susceptible to DNA damage. Donnelly *et al.* (2001) found that cryopreservation of sperm cells from fertile men does not have a deleterious effect on sperm DNA integrity in either unprepared or prepared samples and that the sperm DNA is still suitable for ICSI or IVF. However, with infertile men a significant increase in DNA damaged sperm cells was found. Other studies show that the procedure does not compromise sperm cell DNA integrity (Isachenko *et al.*, 2004; Paasch *et al.*, 2004)

#### **1.7.2.5 Extrinsic factors**

There are various life style factors that have been associated with sperm DNA fragmentation and impaired chromatin integrity. These include diet choice, excessive alcohol consumption, cigarette smoking, caffeine intake, antibiotics, hyperthermia, air pollution irradiation and chemotherapy (Shafik *et al.*, 2006).

Chemotherapy for the treatment of cancer, genital tract inflammation, semen infections, hormonal disorders and age are linked to sperm DNA abnormalities (Shafik *et al.*, 2006; Sakkas and Alvarez, 2010).

#### **1.7.3 Role of oocyte in sperm DNA damage**

Oocytes and early embryos have been shown to be equipped with mechanisms to repair some paternal DNA anomalies (Gasca *et al.*, 2007). Consequently, the biological effect of abnormal sperm structure depends on the combined effects of sperm chromatin damage and the capacity of the oocyte to repair it. The ability of the oocyte to repair depends on the cytoplasmic and genomic quality of the oocyte, which is impacted dramatically by age (Wyrobek *et al.*, 2006).

#### **1.7.4 Assessment of Sperm DNA damage**

Several assays have been developed to evaluate sperm chromatin and DNA integrity. Although each test evaluates sperm cell quality, each may be giving a different aspect of sperm DNA damage and therefore an important aspect in DNA fragmentation analysis is to consider which type of DNA breakage are produced in the DNA strands. Although the mechanism by which the sperm DNA integrity is assessed varies, most tests of sperm DNA integrity correlate well with each other. An important aspect to consider in these tests is their repeatability over time in the same patient.

#### **1.7.4.1 DNA/Chromatin Integrity**

Assays using fluorochromes with specific and complex chromatin/DNA interactions have been developed and these assays identify more subtle defects. These assays include the single cell gel electrophoresis assay (COMET), terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL), sperm chromatin structure assay (SCSA) and acridine orange test [AOT].

##### *Single-cell gel electrophoresis (COMET) assay*

The COMET assay is a test for the direct assessment of sperm cell DNA breaks. Damaged DNA strands separate from intact DNA in the sperm cell head and expand out of the nucleus into agarose gel. The agarose gel is subjected to an electrophoretic gradient and the sperm cell is stained with fluorescent DNA-binding dye and imaged with imaging software (Henkel, 2007). The migration of the DNA strand breaks depends on the intensity of fragmentation – highly fragmented sperm cells will have a greater comet tail size (Schulte *et al.*, 2010). Therefore evaluation involves the length of the comet tail and the amount of DNA in the tail.

##### *Sperm Chromatin Dispersion Test*

The SCD test is a simple and inexpensive assay and measures the absence of damage rather than the damaged DNA in sperm cells. It is based on the principle that sperm cells with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops that are observed on sperm cells with non-fragmented DNA (Fernandez *et al.*, 2005). It does not rely on colour or fluorescence intensity and is simple to analyse with light microscopy.

##### *Terminal Deoxynucleotidyl Transferase-mediated Nick End Labelling (TUNEL)*

The TUNEL assay is a direct quantification of sperm cell DNA breaks. The breaks are labelled with incorporated deoxyuridine triphosphate [dUTP] in a reaction catalysed by the enzyme terminal deoxynucleotidyl transferase [TdT] and can be measured using bright field or fluorescent microscopy as well as flow cytometry. This assay has been regarded as being precise and reproducible (Muratori *et al.*, 2010). A number of different factors such as

preparation, fixation and permeabilization of samples negatively affect its clinical application. Freezing raw or prepared samples does not affect the results of the TUNEL assay (Sailer *et al.*, 1995). The TUNEL and SCSA test correlate very well although they determine different aspects of sperm cell function (Muratori *et al.*, 2010). The TUNEL assay has however not been standardized to the same extent as the SCSA.

#### *Sperm Chromatin Structure Assay (SCSA)*

The SCSA assay is based on the principle that DNA in sperm cells with abnormal chromatin structure is more prone to acid or heat denaturation. Using flow cytometry, it measures the susceptibility of sperm DNA to acid-induced denaturation in situ and determines the extent of DNA damage. Flow cytometry is used to detect and analyse AO fluorescence (Simon *et al.*, 2010). The DNA fragmentation index (DFI) describes the percentage of abnormal sperm cells (red-orange stained sperm) (Fernández *et al.*, 2005; Oleszczuk *et al.*, 2011). The SCSA can be predictive of fertilization failure in vivo and in vitro (Evenson and Wixon; 2006).

#### *Acridine Orange Test (AOT)*

The AOT is a simplified microscopic method of the SCSA that does not require expensive flow cytometric equipment and a SCSA trained technician. Similar to the SCSA, the AOT measures the susceptibility of sperm cell nuclear DNA to acid-induced denaturation and thus measure the stability of sperm cell chromatin (Kazerooni *et al.*, 2009). The Acridine Orange dye binds to the single-stranded DNA and a concentration dependent loss of absorbed energy takes place, causing a metachromatic shift of acridine orange fluorescence from green (native DNA) to red (denatured DNA) (Apedaile *et al.*, 2004).

#### **1.7.4.2 Chromatin Packaging**

Chromatin of mature sperm cells has been shown to have a varying binding capacity for many nuclear dyes and stains. Several factors influence the staining of the sperm cell chromatin, such as the secondary structure of DNA, regularity and density of the chromatin packaging, and binding of DNA to chromatin proteins (Esterhuizen, 2000). Chromomycin-A3 (CMA3) and Aniline Blue (AB) staining techniques have been used as measures of chromatin condensation anomalies. These techniques can potentially identify abnormal chromatin condensation in morphologically normal and abnormal sperm cells

##### *Chromomycin A<sub>3</sub> (CMA<sub>3</sub>)*

CMA<sub>3</sub> is a guanine-cytosine specific fluorochrome and competes with protamines for binding to the minor groove of the DNA helix. The test therefore detects protamine-deficiency in loosely packed chromatin (Ramos et al., 2008). Thus, the degree of CMA<sub>3</sub> staining inversely correlates with the protamination state and directly assesses protamine deficiency (Bizarro et al., 1998). It is also a useful to detect nicked DNA. This test is simple and easily performed in Andrology laboratories. There is a relationship between abnormal protamination and diminished semen quality parameters which is reasonable considering that the regulation of protamine exchange is linked to a broader control of spermatogenesis (Carrell et al., 2007).

##### *Aniline Blue (AB)*

The AB test is used to assess the nuclear maturity of sperm cells. AB selectively stains lysine-rich histones and may be an indication of a lower amount of protamines and immature chromatin condensation. Sperm cells with immature chromatin condensation will stain positive blue, whereas sperm cells containing mature chromatin will not be susceptible to the stain (Kazerooni et al., 2009). AB is indicated to be predictive of fertilization and pregnancy rates following IVF (Hammadeh et al., 1998).

### **1.7.5 Effect on ART Outcome**

Fertilisation, embryo quality and pregnancy rates are all important reproductive parameters that could potentially be affected by sperm DNA damage.

#### **1.7.5.1 Fertilization**

Studies have shown that sperm cell DNA integrity becomes particularly relevant when fertilization occurs in a more natural way (IUI) when compared to in vitro methods (IVF and ICSI) (Duran *et al.*, 2002; Gandini *et al.*, 2004).

If the proportion of DNA damage exceeds 30% (as measured by SCSA), the probability of fertilization with IUI is almost zero (Saleh *et al.*, 2003; Bungum *et al.*, 2004). Several studies have found no significant correlation between sperm cell DNA damage and fertilization rates with IVF or ICSI (Tomlinson *et al.*, 2001; Morris *et al.*, 2002), while other studies have found a negative correlation (Lopes *et al.*, 1998; Host *et al.*, 2000; Schulte *et al.*, 2010).

This difference between in vivo and in vitro fertilisation rates is expected. In ICSI, where the sperm cell is chosen and injected, it has been shown that sperm cells with fragmented DNA can fertilize oocytes with the same efficiency as sperm cells without DNA fragmentation (Twigg *et al.*, 1998). However, if critical genes are damaged when the paternal genome is activated at day 3 (four to eight cell stage), embryo development failure is likely to occur.

#### **1.7.5.2. Embryo quality**

There has been no consistent relationship between sperm DNA damage and embryo quality during IVF cycles. Several studies did not identify any adverse effect of sperm DNA damage on embryo quality (Lopes *et al.*, 1998; Tomlinson *et al.*, 2001; Larson-Cook *et al.*, 2003; Benchaib *et al.*, 2003; Huang *et al.*, 2005) while others reported a negative correlation (Morris *et al.*, 2002; Seli *et al.*, 2004; Virro *et al.*, 2004; Zini *et al.*, 2005). There are also reports of a

correlation between sperm DNA damage and blastulation. There are indications that high levels of DNA damage in a sperm sample may lead to failure to obtain blastocysts (Seli *et al.*, 2004).

Abnormal morphology of sperm cells may have a negative paternal effect on preimplantation embryo development. Defective protamine packaging in sperm DNA and their incorrect replacement by histones during fertilization may create problems such as asynchronous cleavage and delays in the cell cycle after fertilization, thereby lowering blastocyst formation (Shoukir *et al.*, 1998).

#### **1.7.5.3 Pregnancy**

There is a great deal of uncertainty concerning pregnancy rates and DNA fragmentation. Sperm tests of DNA integrity seem to lose their predictive power in respect to reproductive outcomes with ICSI and IVF.

With IUI, semen samples with more than 12% damage (as measured by TUNEL) achieved no pregnancies (Duran *et al.*, 2002). With IVF and ICSI, several studies have evaluated DNA structure and integrity and reported a negative effect of high percentages of sperm cells with damaged DNA on pregnancy rates (Tesarik, 2004). It was reported in multiple studies that no clinical pregnancy could be obtained after IVF or ICSI when the DFI (as measured by SCSA) was more than 20%. Another study showed that DNA fragmentation (as measured by TUNEL) was lower when a pregnancy was obtained using ICSI and no pregnancy was obtained when DNA fragmentation was more than 20% (Duran *et al.*, 2002). However, a more recent study showed no differences in pregnancy rates after IVF or ICSI between patients with high and low levels of DNA fragmentation using a TUNEL value of 15% as a cut-off. It has been shown that pregnancy rates with ICSI were better than with IVF with high levels of sperm DNA damage (DFI > 30%) (Bungum *et al.*, 2004). The study by Bungum *et al.* (2004) found higher pregnancy rates and delivery rates with ICSI compared to IVF when samples with high DNA



damaged sperm cells were used. Most reports find that sperm DNA damage is more predictive in IVF and less in ICSI.

#### **1.7.5.4 Offspring**

Focus on the genomic material of the male has been intensified due to growing concern about transmission of genetic diseases through ICSI (Agarwal *et al.*, 2003). The ICSI procedure, which bypasses natural selection, was initially developed for men with impaired semen parameters. ICSI can lead to pregnancy regardless of traditional sperm cell quality parameters and sperm chromatin structural integrity. This may lead to potential chromosomal abnormalities in ICSI-born progeny (Erenpreiss *et al.*, 2006)

### **1.8 THE RESEARCH QUESTION**

Semen samples are incubated at different times and temperatures before and after the sample is processed. IVF samples are usually incubated at 37°C and ICSI samples at room temperature [RT]. No consensus has however been reached on what the ideal temperature and time should be to optimize the ART outcome. IVF samples are also collected closer to the insemination time and therefore are incubated for shorter periods compared to ICSI samples. The experimental study aims to establish if there is an effect on the parameters and DNA profile of the sperm cells before and after the semen sample is processed. The retrospective study is independent from the prospective study and examines the incubation time of the processed sample and the effect on the ART outcome. The two studies combined will revise and help establish cut-off times and temperatures at which damage to the sperm cell is at a minimum.

## 1.9. AIMS AND OBJECTIVES

In this study, the primary objective was to evaluate the impact of various storage time intervals and temperature on motility and the DNA profile of sperm cells. This evaluation was done on two different populations – for the semen samples before processing one group of men were used and another group of men for the processed sample. Due to the logistics of the analysis it was not possible to use the same sample for both evaluations. The CMA<sub>3</sub> assay was applied to determine the chromatin maturity and compaction and the TUNEL assay to assess the level of DNA fragmentation.

The secondary objective was to retrospectively assess what the effect of sperm cell incubation time before insemination has on ART outcome (fertilization rates, embryo quality and pregnancy rates).

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1 PRIMARY OBJECTIVE**

#### **2.1.1 Study Population**

This prospective analytical study was conducted at the Andrology/Fertility clinics at Tygerberg and Drs Aevitas Fertility Clinic (Vincent Pallotti Hospital) in Cape Town, South Africa between June 2013 and October 2014. The Health Research Ethics Committee (HREC) of the University of Stellenbosch approved the study protocol (REF nr: S13/05/113). Samples were obtained from routine semen analysis and, if possible, from ART patients. Excess semen that is usually discarded after semen analysis was used in this study and therefore a waiver of consent was granted. Overall, 78 samples were obtained – 40 in the non-processed group and 38 in the processed group. Four samples were excluded from the pre-preparation group (n=36) and five from the post-preparation group (n=33) due to unreadable CMA<sub>3</sub> or TUNEL tests.

#### **2.1.2 Sample Collection and Evaluation**

Semen specimens were obtained by masturbation and only samples that were produced at the clinic were included in this study. This allowed for better monitoring of the time and temperature at which the sample was produced. Semen specimens were allowed 20 minutes for liquefaction at 37°C. After liquefaction, samples were analysed for sperm count, motility and forward progression (Addendum I). Semen smears for sperm morphology were made according to the WHO guidelines (WHO, 2010) and stained using the diff-quick [D-Q] staining method (Addendum II).

### 2.1.3 Semen Evaluation, Preparation and Incubation

Semen samples were assigned to either study 1 or study 2. Study 1 focused on the incubation of the non-processed semen sample and study 2 on the incubation of the processed semen sample.

#### *Study 1: Raw, non-processed semen samples*

Semen samples with a concentration of more than  $15 \times 10^6$  sperm/ml and a motility of more than 30% were included. Specimens with a high viscosity and low volume (<1ml) were excluded. Each sample was equally divided into 6 tubes (RT/20; 37°20; RT/40; 37°C/40; RT/60; 37°C/60). Three tubes were incubated at RT for 20, 40 and 60 minutes respectively and the other three at 37°C for 20, 40 and 60 minutes respectively (Figure 2.1). RT was measured and noted at the time of incubation. After the incubation period, each sample was evaluated (count, motility forward progression), a smear was made for a standard CMA<sub>3</sub> staining test (Addendum VI) and a portion ( $\pm 30\mu\text{l}$ ) was frozen for TUNEL evaluation at a later stage (Addendum V).

#### *Study 2: Post-processed semen samples*

Semen samples with a concentration of more than  $20 \times 10^6$  sperm/ml and a motility of more than 30% were included. Specimens with a high viscosity and low volume (<1ml) were excluded. After liquefaction and evaluation, the sample was prepared by the swim-up procedure to obtain washed, motile sperm cells (Addendum III).

After the sample was processed, a smear was made for the CMA<sub>3</sub> staining test and each sample was equally divided into 6 aliquots (RT/30; 37°C/30; RT/60; 37°C /60; RT/90; 37°C/90). Three tubes were incubated at RT for 20, 40 and 60 minutes respectively and the

other three at 37°C for 30, 60 and 90 minutes respectively (Figure 2.2). RT was measured at the time of incubation. After the incubation periods, the sample was evaluated (count, motility and forward progression), a smear was made for a standard CMA<sub>3</sub> staining test (Addendum VI) and a portion ( $\pm 30\mu\text{l}$ ) was frozen for TUNEL evaluation at a later stage (Addendum V).

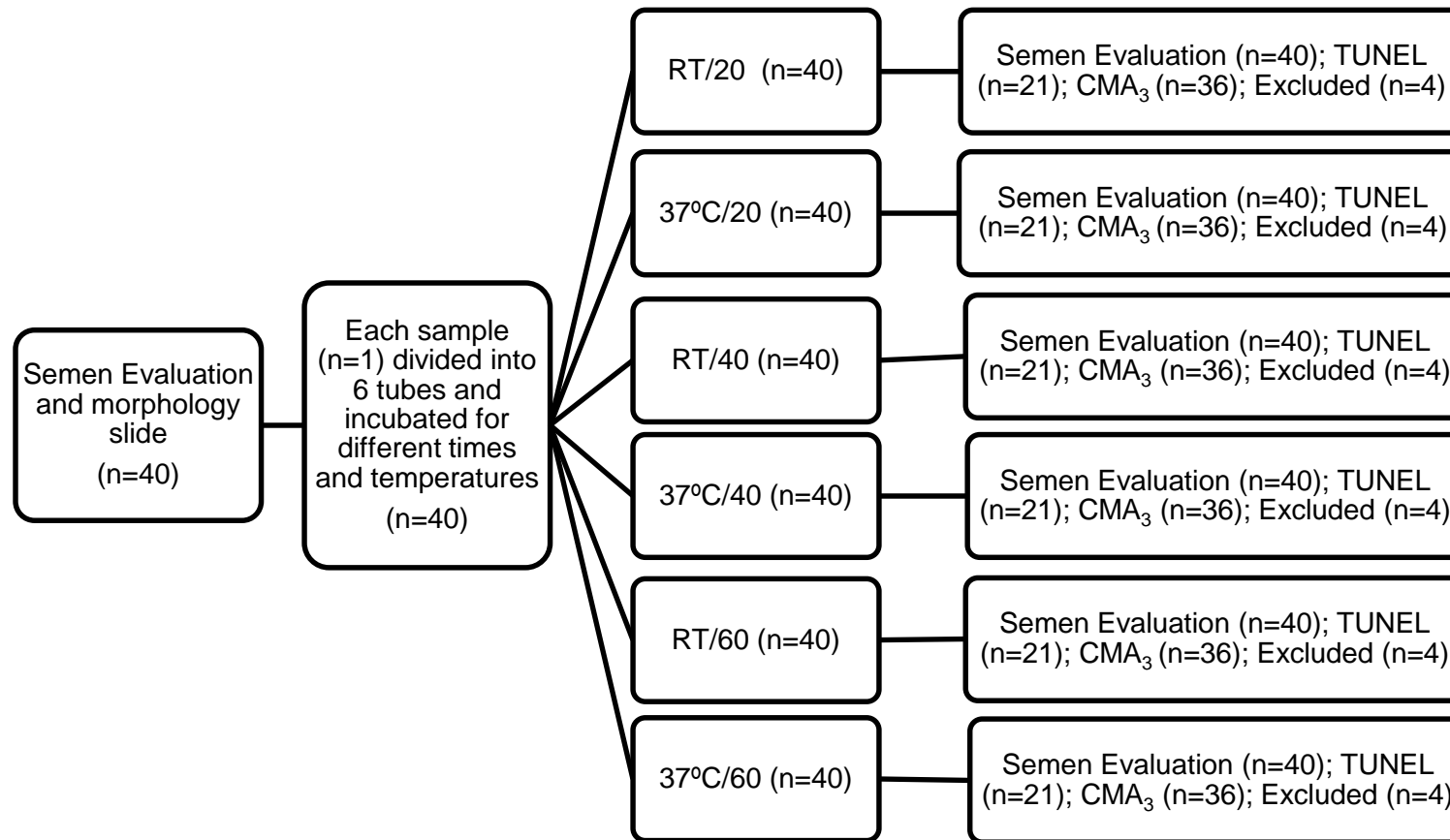


Figure 2.1: Study 1: The incubation of non-processed semen samples at different times and temperatures

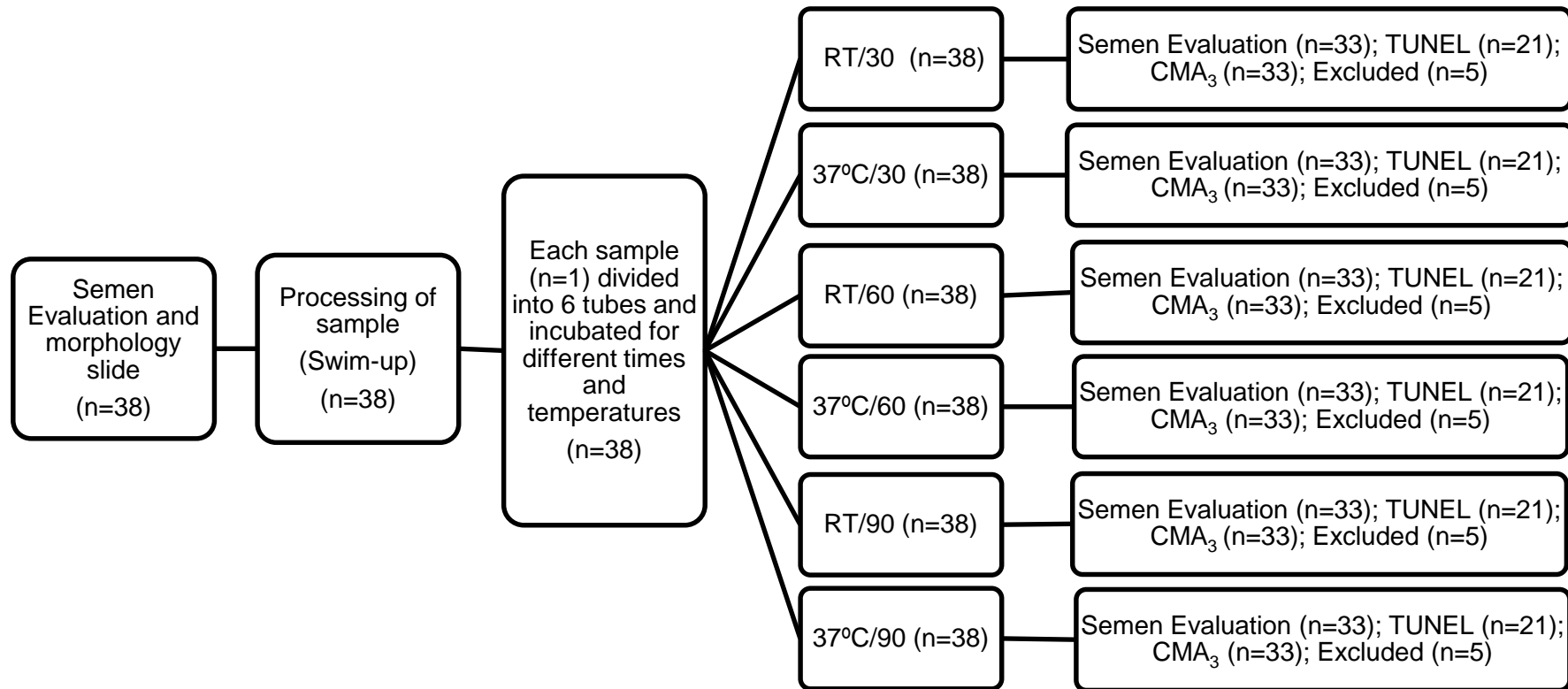


Figure 2.2: Study 2: The incubation of processed semen samples at different times and temperatures

#### 2.1.4 CMA<sub>3</sub> Procedure

Sperm chromatin packaging and maturity was assessed using the Chromomycin A<sub>3</sub> method (Addendum VI).

Following preparation of the smears, the slides were fixed in methanol/acetic acid 3:1 acid at RT for 20 minutes. The air-dried fixed smears were treated with 15µl of CMA<sub>3</sub> (Sigma-Aldrich, Johannesburg, South Africa) while kept in a dark chamber for 20 minutes. After staining, the slides were rinsed in McIlvaine's Buffer and mounted with Dabco anti-fade solution (Sigma-Aldrich, Johannesburg, South Africa). The slides were left overnight in a dark chamber and counted the following day on an Olympus BX40 fluorescence microscope (Wirsam Scientific, Cape Town, South Africa) with a 100x oil immersion objective and the appropriate filters.

A total of 100 spermatozoa were randomly assessed for each slide. Spermatozoa with bright yellow staining (CMA<sub>3</sub> positive) were regarded as immature spermatozoa with decondensed chromatin. Dull yellow spermatozoa were regarded as good chromatin packaging and therefore considered mature. Chromatin packaging was considered normal for CMA<sub>3</sub> values of ≤40%. Uncertain abnormal chromatin packaging occurred when CMA<sub>3</sub> values were between 41-60%. Sperm chromatin packaging was considered abnormal for CMA<sub>3</sub> values ≥61% (Esterhuizen *et al.*, 2000). The CMA<sub>3</sub> values were expressed as % positive (% DNA abnormal) sperm cells



### 2.1.5 TUNEL Procedure

Sperm DNA fragmentation was evaluated with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-fluorescein nick end labelling (TUNEL) assay with the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, GmbH, Mannheim, Germany). This assay uses fluorescein-dUTP to label sites of DNA fragmentation (Addendum V).

After each temperature/time interval, a portion of each sample from each incubation group was frozen in a custom designed device (Addendum V) by dunking the device into liquid nitrogen. To perform the TUNEL test, the samples were thawed at 37°C for 10 minutes and each sperm suspension was aliquoted on a multi-well slide (Teflon Printed Diagnostic Cells) and air-dried for 60 minutes. The slide was then fixed with 4% formaldehyde for 45 minutes in a humidified chamber at RT. After fixation, the slides were washed in 0.1% HSA/PBS and then treated with 0.1% Triton X-100/PBS for 10 minutes in a humidified chamber at RT. After Triton-X permeabilization, the slides were washed twice with 1% HSA/PBS. The TUNEL reaction mixture was added and the sperm was incubated for 90 minutes in a dark, humidified chamber at 37°C. After final washes, a drop of Dabco anti-fade solution (Sigma-Aldrich, Johannesburg, South Africa) was added and the slide was covered with cover slips. The slide was analysed immediately after preparation with an Olympus BX40 fluorescence microscope (Wirsam Scientific, Cape Town, South Africa) under a 100x oil immersion objective. The percentage of green fluorescing sperm (TUNEL positive) was determined. The sperm was considered normal for TUNEL values  $\leq 30\%$ . A TUNEL value of  $> 30\%$  indicated fragmented sperm DNA (Henkel *et al.*, 2003; Henkel *et al.*, 2004). The TUNEL values were expressed as % positive (% DNA abnormal) sperm cells.

### **2.1.6 Statistical Analysis**

Statistical analysis was performed using STATISTICA 12.0 software (StatSoft Inc., TULSA, OK, USA). Descriptive statistics was performed on all the parameters. Results are expressed as median (upper quartile – lower quartile). The variance between the groups was estimated using LS mean graphs and the LSD test. The data was tested for normality using the raw residual normality plot. The term “statistically significant” was used to denote a P-value being less than 0.05.

## **2.2 SECONDARY OBJECTIVE**

### **2.2.1 Study Population**

This was a retrospective study looking at incubation times of prepared semen samples before insemination. This study was approved by the Health Research Ethical Committee (HREC) of Stellenbosch (S13/05/113). We reviewed records of patients that have undergone ICSI or IVF cycles at Drs Aevitas Fertility Clinic (Vincent Pallotti Hospital, Cape Town, South Africa) between January 2010 and December 2012 (n=904). Among them, 212 patients underwent IVF treatment and 688 ICSI treatments. Patients were excluded based on the following: 1) less than 3 oocytes at the time of insemination; 2) testis biopsy cycles 3) PGD done on day 3 4) both IVF and ICSI performed and 5) missing information.

### **2.2.2 Ovarian Stimulation and Oocyte Collection**

Female patients underwent controlled ovarian stimulation using standard, routine methods. The stimulation was monitored by ultrasound and E2 measurements; an hCG injection was given when follicles had a mean diameter of >18mm. Oocyte retrieval was performed using standard, routine methods (vaginal ultrasound probe) approximately 36 hours after the hCG injection.

### **2.2.3 Semen Evaluation and Preparation**

The semen sample was prepared for insemination through either the swim-up (Addendum III) or density gradient (Addendum IV) methods, depending on the quality (concentration, motility and forward progression) of the sperm. Sperm preparation buffer (SAGE, Cooper Surgical, Ferring, South Africa) was used for washing and SilSelect (FertiPro, Harrilabs, South Africa) for gradient centrifugation. At Drs Aevitas, there is no standard protocol for the temperature at which the sample should be incubated before and after it has been processed. The sample is predominantly incubated at 37°C before preparation. Depending on the fertilization technique,

the processed semen sample will be incubated after to await insemination/injection at RT (ICSI) or 37°C (IVF). The temperature is not routinely measured therefore temperature is not included in this study,

## 2.2.4 Fertilization Techniques

In vitro fertilization techniques (IVF and ICSI) were done according to standard routine protocols and procedures at Drs Aevitas Fertility Clinic.

### ***Mediums***

Mediums from SAGE In-Vitro Fertilization Inc., Cooper Surgical Company, Trumbull, CT, were used.

For oocyte pick up [OPU] and the ICSI procedure, HEPES buffered Flushing medium were used. Oocytes were incubated in Quinns Advantage Protein Plus Fertilization medium and transferred on day 1 (16-18 hours post-insemination) to Quinns Advantage Protein Plus cleavage medium. On day 3 and 5 (70-72 hours and 120 hours respectively post-insemination), the embryos were transferred to Quinns Advantage Blastocyst Medium Trumbull, CT). Tissue oil (SAGE In-Vitro Fertilization Inc., Cooper Surgical Company, Trumbull, CT) was used for oil overlay and for semen preparation sperm washing medium (SAGE In-Vitro Fertilization Inc., Cooper Surgical Company, Trumbull, CT) was used. Semen preparation by gradient centrifugation was done using SilSelect Media (Fertipro, Beernem, Belgium).

### ***IVF***

Using the routine standard protocol at Drs Aevitas Fertility Clinic, mature MII oocytes were inseminated with 500 000 – 1 000 000 sperm cells in a 4 well NUNC dish (Addendum VIII).

### ***ICSI***

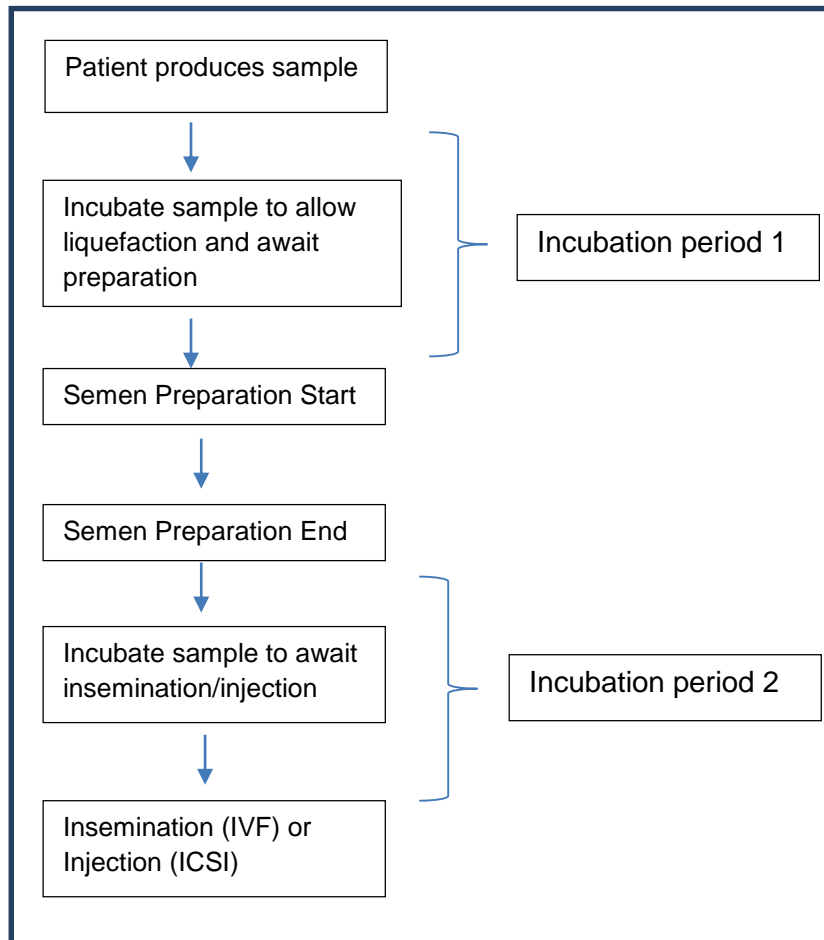
Mature, denuded MII oocytes were injected with a single, immobilized sperm cell, according to a routine standard ICSI protocol (Addendum IX).

### **2.2.5 Timing**

Different time intervals were analysed in this retrospective study (Figure 2.3). The incubation time between sperm preparation and insemination/injection (incubation period 2) was the main focus point of the retrospective study. The data was categorized in 5 groups according to incubation time: group 1 with an incubation time of 1 hour or less, group 2 between 1 and 2 hours, group 3 between 2 and 3 hours and group 4 an incubation time of more than 4 hours.

### **2.2.6 Assessment of Fertilization and Embryo Quality**

Assessment of fertilization was done 16-18 hours after insemination/injection. The presence of 2 pronuclei and 2 polar bodies represented successful fertilization. The fertilization rate was defined as the ratio between the number of fertilized zygotes and the number of mature oocytes. Embryo quality was assessed morphologically on day 2, 3, and 5. Embryos were incubated individually in drops and transferred daily to fresh protein plus cleavage or blastocyst medium. Embryos were regarded as “good quality” when they were at 2-4 cell stage on day 2 (48 hours post-insemination) or at the 6-8 cell stage on day 3 (72 hours post-insemination). The embryos were given a score on a scale of 1-5 (1 is worst and 5 is best morphology) according to blastomere morphology, percentage fragmentation and blastocyst formation (Addendum X). Blastocysts were evaluated on day 5 according to the degree of expansion, the development of an inner cell mass and the appearance of the trophectoderm (Addendum XI)... A “good quality” day 5 blastocyst was defined as having an expansion grading of 2 or 3 with an A or B inner cell mass and trophectoderm grading.



**Figure 2.3: A Schematic representation of the timings of sperm incubation**

### 2.2.7 Embryo Transfer and establishment of Pregnancy

The best quality embryo(s), based on morphology, was selected for transfer. Embryos were transferred into the uterus (trans-vaginal transfer) on day 2, day 3 or day 5 under ultrasound guidance using an Efficiere Series Transfer catheter (Cooper surgical). According to the protocol at Drs Aevitas, a maximum of three embryos are transferred per cycle. In this study, a demonstration of a positive serum  $\beta$ hCG (IU/ml) ( $>10$  IU/ml) was considered a successful establishment of pregnancy. The Drs Aevitas Fertility clinic is mainly a referral clinic and it is therefore difficult to obtain clinical (gestational sac and fetal heartbeat) pregnancy results. It

was especially difficult to find clinical pregnancy results of patients from 2010 and international patients. The high incidence of missing data therefore made it impossible to use clinical pregnancy as an outcome.

### **2.2.8 Outcomes analyzed**

The effects that different semen exposure times might have on ART outcome were analysed in this retrospective study. The outcomes of interest included: 1) IVF and ICSI fertilization rates 2) embryo quality and 3) pregnancy rates.

### **2.2.9 Statistical analysis**

Statistical analysis was performed using STATISTICA 12.0 software (StatSoft Inc., TULSA, OK, USA). Descriptive statistics was performed on all parameters. Results are expressed as mean  $\pm$ SD. Data were represented in Box and Whisker plots (whereby boxes depict the 25<sup>th</sup> and 75<sup>th</sup> percentiles with indication of the median value and whiskers depict the 10<sup>th</sup> and 90<sup>th</sup> percentiles) and Lowess Smoother plots. The relationship between the parameters was examined using the Mann-Whitney U Test and an  $\chi^2$ -test was used to assess the parameters and the different incubation time groups. The relationship was also examined using linear regression techniques. The term “statistically significant” was used to denote a P-value being less than 0.05.



## CHAPTER 3: RESULTS

### 3.1 PRIMARY OBJECTIVE 1:

#### THE EFFECT OF INCUBATION TIME AND TEMPERATURE ON SPERM MOTILITY AND DNA OF THE NON-PROCESSED SEMEN SAMPLE

This study included 40 samples – 4 were excluded due to unreadable CMA<sub>3</sub> tests.

An overview of the outcomes after the different temperature and incubation times are summarized in Table I. The mean RT at which the samples were incubated was 23.93±1.009°C.

The median of the semen count and motility before incubation was 40(30-70)10<sup>6</sup>/ml and 55(50-60)% respectively. After incubation, the median (overall for all temperatures and incubation times) of the count remained 40(30-60)10<sup>6</sup>/ml as expected and the motility decreased to 50(50-60)% . The count (10<sup>6</sup>/ml) remained relatively constant over the different temperatures and incubation time intervals. No significant difference as determined by an x<sup>2</sup>-test (as expected) was found between the count incubation groups (P=0.072) indicating that the samples were accurately and evenly divided for all groups. The median CMA<sub>3</sub> and TUNEL value (after incubation - overall for all temperatures and incubations) was 11(8-15.5)% and 13(10-15)% respectively. The median normal morphology value was 9.5 (4.5-11)% with 86.11% of the samples in the ≥4% and <15% normal sperm morphology group; 8.83% in the <4% and 5.56% in the ≥ 15% normal morphology groups. The detailed, different incubation group outcomes are summarized in Table II.

**Table I: Median (upper-lower quartile range) values of the sperm parameters of the non –processed semen sample when incubated at different times and temperatures before and after incubation**

	N	BEFORE INCUBATION <sup>b</sup>	AFTER INCUBATION				
			Temperature (°C)		Incubation time (minutes)		
			RT	37	20	40	60
<b>Normal Morphology (%)</b>	36	9.5(4.5-11)	a	a	a	a	a
<b>Count (10<sup>6</sup>/ml)</b>	36	40(30-70)	40(30-60)	40(30-70)	47.5(30-62.5)	40(30-70)	40(30-65)
<b>Motility (%)</b>	36	55(50-60)	50(50-60)	55(50-60)	55(50-60)	55(50-60)	55(50-60)
<b>CMA<sub>3</sub> (%)<sup>c</sup></b>	36	a	11(8-15.5)	11(8-16)	10(7-15)	10(8-16)	13(8-17.5)
<b>TUNEL (%)<sup>c</sup></b>	21	a	11(8-14)	13.5(10-18)	10(7-13)	10(8-12.75)	13.5(10-18)

<sup>a</sup>Parameter was not assessed

<sup>b</sup>Before incubation temperature: 37°C

<sup>c</sup>Percentage positive (% DNA abnormal) sperm

**Table II: Median (lower-upper quartile range) values of the sperm parameters of the non-processed semen sample in the different incubation groups before and after incubation**

	N	BEFORE INCUBATION <sup>b</sup>	AFTER INCUBATION						P-VALUE <sup>d</sup>
			RT/20	37°C/20	RT/40	37°C/40	RT/60	37°C/60	
<b>Normal Morphology (%)</b>	36	9.5(4.5-11)	a	a	a	a	a	a	a
<b>Count (10<sup>6</sup>/ml)</b>	36	40(30-70)	50(27.5-65)	42.5(30-62.5)	40(30-60)	40(30-70)	40(30-60)	40(25-70)	0.072
<b>Motility (%)</b>	36	55(50-60)	50(50-60)	55(50-62.5)	52.5(50-60)	55(50-62.5)	52.5(45-60)	55(50-60)	0.013
<b>CMA<sub>3</sub> (%)<sup>c</sup></b>	36	a	10(7-15)	10(7-15.5)	10(8-15.5)	10(7-16.5)	13(7.5-17.5)	14(8.5-17.5)	0.27
<b>TUNEL (%)<sup>c</sup></b>	21	a	9.5(6.5-13.5)	10(7-12)	8(10-12)	8(10-15)	14(12-15)	11(9-14)	0.17

<sup>a</sup>Parameter was not assessed

<sup>b</sup>Before incubation temperature: 37°C

<sup>c</sup>Percentage positive (% DNA abnormal) sperm

<sup>d</sup>P-value of <0.05 is considered significant

### 3.1.1. Effect of Incubation Time and Temperature

#### 3.1.1.1 Motility – non-processed sample

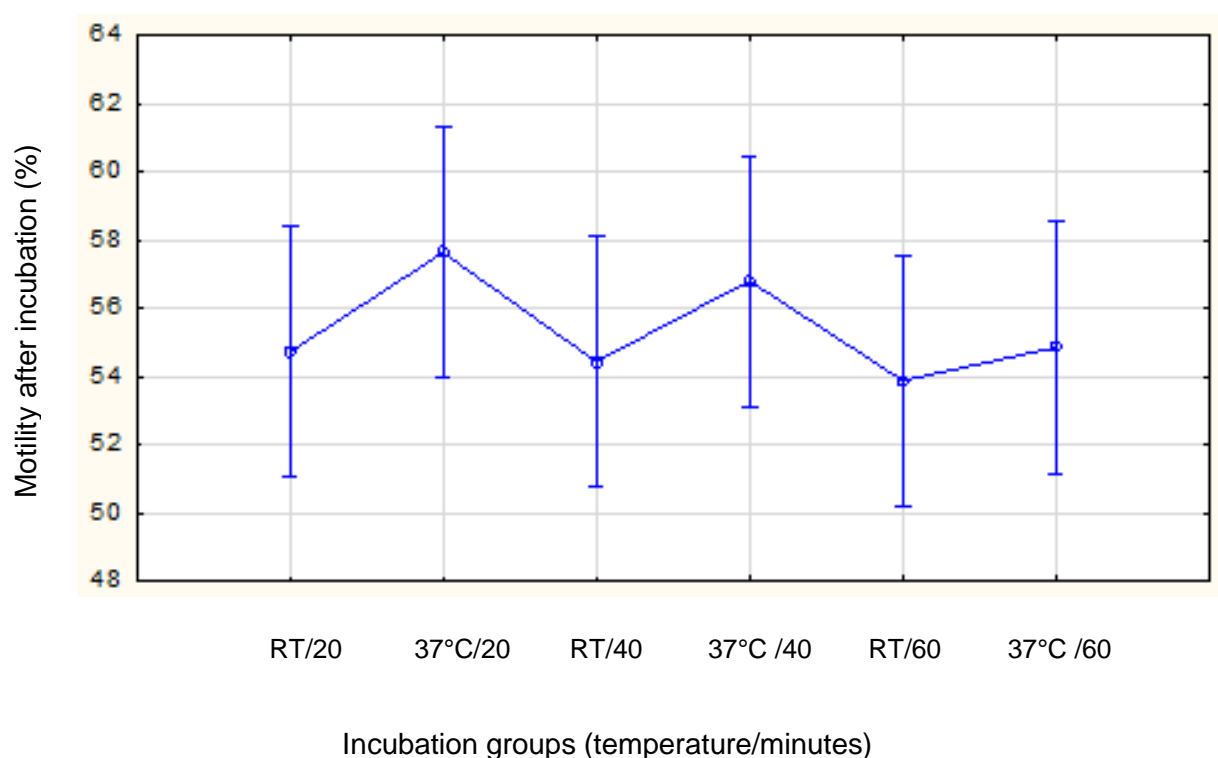
The effect of incubation time and temperature on sperm motility is presented in Table II, Table III and Figure 3.1(n=36).

The motility (%) of the sperm appeared to be relatively stable between the groups [RT/20: 50(50-60); 37°C/20: 55(50-62.5); RT/40: 52.5(50-60); 37°C/40: 55(50-62.5); RT/60: 52.5(45-60); 37°C/60: 55(50-60)] (Table II). However, statistically, a significant difference was observed between individual incubation groups and the motility of the sperm ( $P=0.013$ ). Table III highlights (in red) these differences. When the LS Means were compared (Figure 3.1), the significant effect was apparent in the 37°C/20 group, which differed significantly ( $P<0.05$ ) from most of the other groups (RT/20, RT/40, RT/60 and 37°C/60) and had a positive effect on the sperm motility. The 37°C/40 group also showed a significantly ( $P<0.05$ ) higher motility compared to the RT/60 group (Table III).

**Table III Post Hoc test displaying where the significance in motility (%) in the LS means (figure 3.1) between the different incubation groups of the non-processed semen sample lie**

	RT/20min	37°C/20min	RT/40min	37°C/40min	RT/60min	37°C/60min
RT /20min		0.017148	0.818990	0.087398	0.492636	0.908898
37°C /20min	0.017148		0.009151	0.492636	0.002299	0.023103
RT/ 40min	0.818990	0.009151		0.053002	0.647250	0.731422
37 °C/ 40min	0.087398	0.492636	0.053002		0.017148	0.110446
RT /60min	0.492636	0.002299	0.647250	0.017148		0.423548
37 °C /60min	0.908898	0.023103	0.731422	0.110446	0.423548	

Red colour indicate a significant P value ( $< 0.05$ )

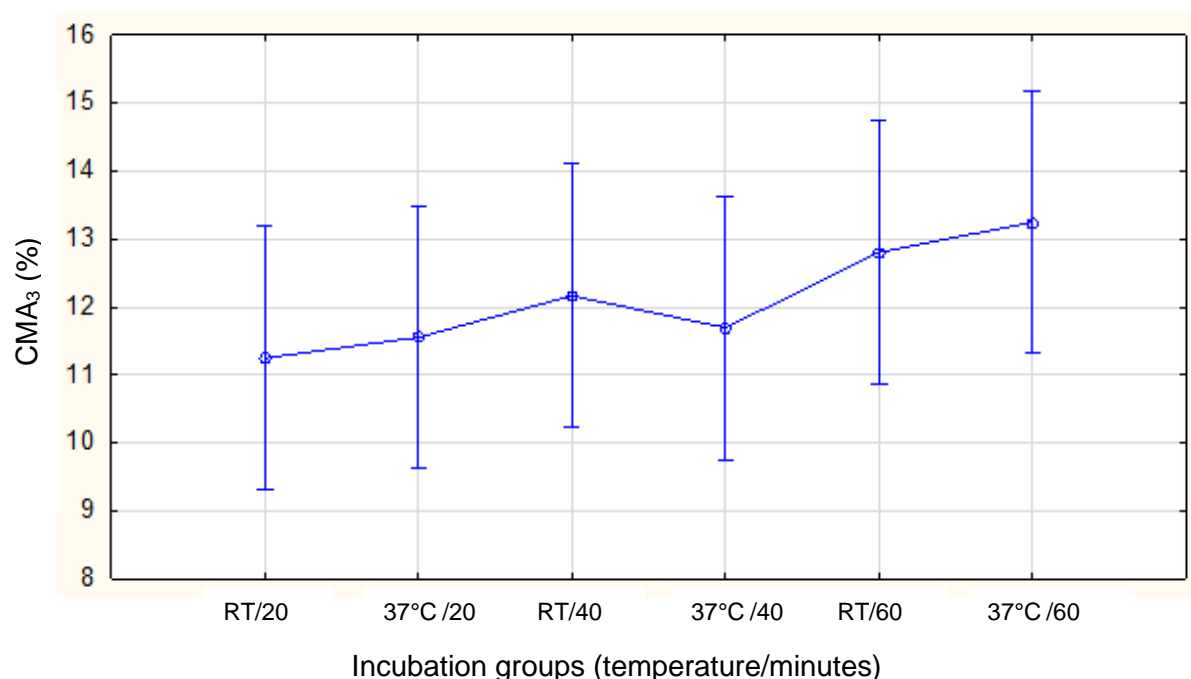


**Figure 3.1: The LS means of the motility after incubation of the non-processed semen sample (n=36) in the 6 incubation groups ( $p < 0.05$ )**

### 3.1.1.2. CMA<sub>3</sub> – non-processed sample

The effect of incubation time and temperature on sperm CMA<sub>3</sub> is presented in Table II and Figure 3.2 (n=36). The CMA<sub>3</sub> values are expressed as percentage positive (% DNA abnormal) sperm. The values ranged between 2 and 30%, falling in the normal range (<40% positive sperm cells).

The CMA<sub>3</sub> values (%) seemed to increase over time [RT/20: 10(7-15); 37°C/20: 10(7-15.5); RT/40: 10(8-15.5); 37°C/40: 10(7-16.5); RT/60: 13(7.5-17.5); 37°C/60: 14(8.5-17.5)] (Table II). The 37°C/60 group appeared to have the worst chromatin packaging profile – highest CMA<sub>3</sub> score (Figure 3.2). However, statistically, no significant difference was found (P=0.27) between the groups.

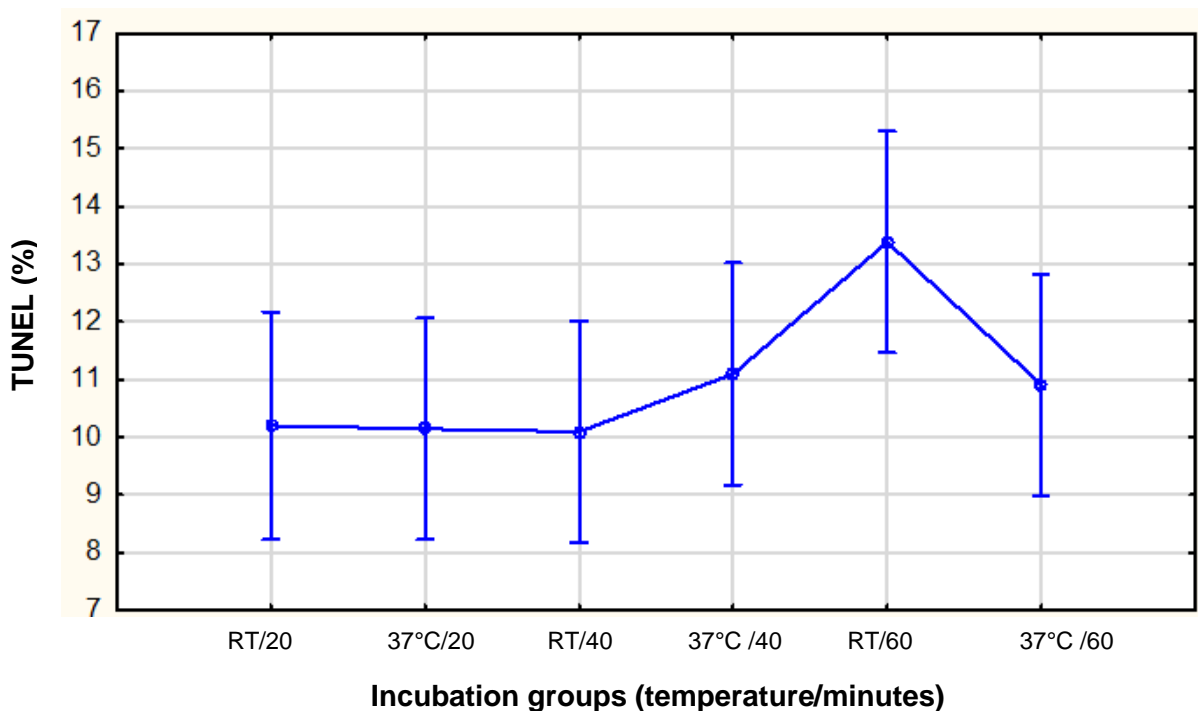


**Figure 3.2: The LS means of the CMA<sub>3</sub> values after incubation of the non-processed semen sample (n=36) in 6 incubation groups (p>0.05)**

### 3.1.1.3. TUNEL – non-processed sample

The effect of incubation time and temperature on sperm DNA fragmentation [TUNEL] is presented in Table II and Figure 3.3 (n=21). The TUNEL values are expressed as percentage positive (% DNA abnormal). The values ranged between 1 and 23%, falling in the normal range ( $\leq 30\%$  positive sperm cells).

The TUNEL (%) values varied between the different groups [RT/20: 9.5(6.5-13.5); 37°C/20: 10(7-12); RT/40: 8(10-12); 37°C/40: 8(10-15); RT/60: 14(12-15); 37°C/60:11(9-14)] (Table II). From Figure 3.3, it appeared that the RT/60 group had increased TUNEL values. There was however no statistical significant difference between the TUNEL values in the different incubation groups ( $P=0.17$ ).



**Figure 3.3: The LS means of the TUNEL values after incubation of the non-processed semen sample (n=21) in 6 incubation groups ( $p>0.05$ )**

#### *3.1.1.4. TUNEL and CMA3 correlation – pre-preparation*

A fixed effect test using a restricted maximum likelihood found no association between TUNEL and CMA<sub>3</sub> ( $p=0.058$ ). All the TUNEL and CMA<sub>3</sub> values also fell within the normal range.

### 3.1.2. Summary of Prospective Study 1 (before semen preparation)

- Incubation time and temperature had a significant effect on the motility of the sperm
  - 37°C incubations resulted in higher motility relative to the RT incubations
  - Highest motility was seen in the 37°C/20 group
  - The 37°C/40 group had a significantly higher motility than the RT/60 group
- Incubation time and temperature did not have a significant effect on the CMA<sub>3</sub> or the TUNEL results
  - There was an increase over time in the CMA<sub>3</sub> values
  - Higher TUNEL values were seen at RT/60
- No association between TUNEL and CMA<sub>3</sub> was found



### 3.2. PRIMARY OBJECTIVE 2:

#### STUDY 2: THE EFFECT OF INCUBATION TIME AND TEMPERATURE ON SPERM MOTILITY AND DNA OF THE PROCESSED SEMEN SAMPLE

This study included 33 semen samples. 5 samples were excluded due to unreadable CMA<sub>3</sub> or TUNEL slides.

An overview of the outcomes of different temperature and incubation times before and after the sample was processed is summarized in Table IV. The mean RT between the different incubation groups was  $21.34 \pm 1.17^{\circ}\text{C}$ .

The median of the semen count and motility before swim-up was 70(35-100)  $10^6/\text{ml}$  and 55(50-60)% respectively. After swim-up, the median for the count decreased to 30(15-60)  $10^6/\text{ml}$  and motility increased to 90(85-90)%. The effect of incubation time and temperature of the processed sample on sperm count is presented in Table IV and V. The count ( $10^6/\text{ml}$ ) remained relatively consistent over the different temperatures and incubation time intervals (Table IV) and also remained stable between the different incubation groups (Table V). No significant difference (as expected) was observed between the different groups ( $p=0.18$ ), indicating that the samples were accurately and evenly divided for all groups.

The median normal morphology value was 9.0 (6-13) % with 69.70% of the samples falling in the  $\geq 4\%$  and  $< 15\%$  normal sperm morphology group; 15.15% under the  $< 4\%$  group and 15.15% under  $\geq 15\%$  normal morphology group. The detailed, different incubation group outcomes are summarized in Table V.

**Table IV: Median (upper-lower quartile range) values of the sperm parameters over different temperatures and times before and after the sample was processed and incubated**

	N	BEFORE PREPARATION <sup>b</sup>	AFTER PREPARATION	AFTER INCUBATION				
				Temperature (°C)		Incubation time (minutes)		
				RT	37	30	60	90
<b>Normal Morphology (%)</b>	33	9.00(6-13)	a	a	a	a	a	a
<b>Count (10<sup>6</sup>/ml)</b>	33	70(35-100)	30(15-60)	40(20-60)	40(20-70)	35(20-70)	40(20-60)	40(20-60)
<b>Motility (%)</b>	33	55(50-60)	90(85-95)	85(75-90)	85(80-95)	90(80-95)	85(75-95)	82.5(75-90)
<b>CMA<sub>3</sub> (%)<sup>c</sup></b>	33	a	15(6-25)	14(9-24)	15(8-22)	14(7-19)	15(9-25)	13(8-25)
<b>TUNEL (%)<sup>c</sup></b>	20	a	a	13.5(10-18)	14(9-17)	14(9.75-17.25)	12(10-17.5)	14(10-17.25)

<sup>a</sup>Parameter was not assessed<sup>b</sup>Temperature before preparation: 37°C<sup>c</sup>Percentage positive (% DNA abnormal) sperm**Table V: Median (upper-lower quartile range) values of the sperm parameters between the different incubation groups before and after the sample was processed and incubated**

	N	BEFORE PREPARATION <sup>b</sup>	AFTER PREPARATION	AFTER INCUBATION						P-VALUE <sup>d</sup>
				RT/30	37°C/30	RT/60	37°C/60	RT/90	37°C/90	
<b>Normal Morphology (%)</b>	33	9.00(6-13)	a	a	a	a	a	a	a	a
<b>Count (10<sup>6</sup>/ml)</b>	33	70(35-100)	30(15-60)	40(20-70)	30(20-60)	40(20-60)	40(20-70)	40(25-60)	40(20-60)	0.18
<b>Motility (%)</b>	33	55(50-60)	90(80-95)	90(75-90)	90(80-95)	85(75-90)	85(80-95)	80(75-90)	85(75-95)	0.018
<b>CMA<sub>3</sub> (%)<sup>c</sup></b>	33	a	15(6-25)	14(9-18)	14(6-20)	17(8-28)	15(9-20)	12(9-25)	15(8-25)	0.18
<b>TUNEL (%)<sup>c</sup></b>	20	a	a	14(9.5-17)	13(9.5-18.5)	12(11-18.5)	14(7-16)	14(9.5-19)	14(10.5-16)	0.95

\*Parameter was not assessed

<sup>b</sup>Temperature before preparation: 37°C<sup>c</sup>Percentage positive (% DNA abnormal) sperm<sup>d</sup>P-value of <0.05 is considered significant

### 3.2.1 Effect of Incubation Time and Temperature

#### 3.2.1.1. Motility – *processed sample*

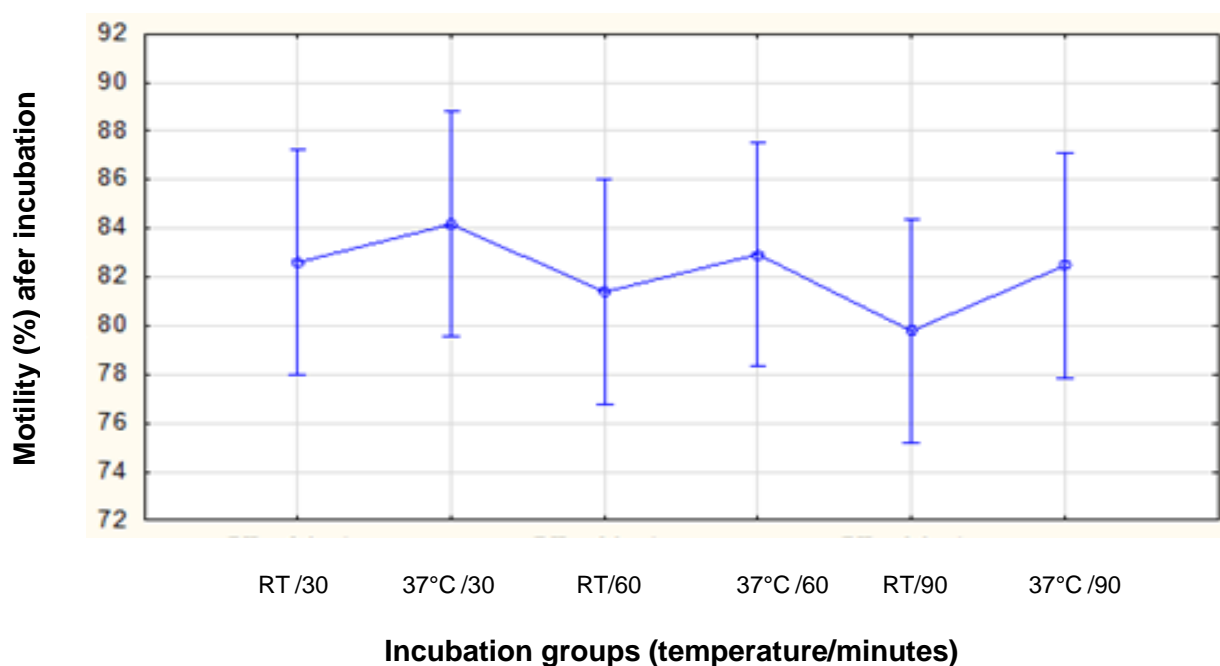
The effect of post-preparation incubation time and temperature on sperm motility is presented in Table V, Table VI and Figure 3.4 (n=33).

The motility (%) did appear to differ between the groups [RT/30: 90(75-90); 37°C/30: 90(80-95); RT/60: 85(75-90); 37°C/60: 85(80-95); RT/90: 80(75-90); 37°C/90: 85(75-95)] Table V). The fixed effect test found a significant difference between the groups ( $P=0.018$ ) (blocks highlighted in red, Table VI). The LS means displayed in Figure 3.4 depict the decrease of motility over time. The RT/90 group (LS Mean:  $74.00 \pm 4.21$ ) showed a significant decrease in motility compared to most other groups. Statistically there was no significant difference between the RT/60 and RT/90 groups. There was significantly less motility in RT/60 compared to 37°C/30min group ( $P = 0.029$ ) (Table VI).

**Table VI: Post hoc test displaying where the significant difference in the motility (%) (Figure 3.4) between the different incubation groups of the processed semen sample lie**

	RT/30min	37°C/30min	RT/60min	37°C/60min	RT/90min	37°C/ 90min
RT/30min		0.214588	0.339279	0.792446	0.027247	0.923763
37°C/30min	0.214588		0.028920	0.327377	0.000659	0.181550
RT/60min	0.339279	0.028920		0.223493	0.205945	0.389643
37°C/60min	0.792446	0.327377	0.223493		0.013722	0.719754
RT/90min	0.027247	0.000659	0.205945	0.013722		0.034486
37°C/90min	0.923763	0.181550	0.389643	0.719754	0.034486	

Red indicates a significant P-value (< 0.05)

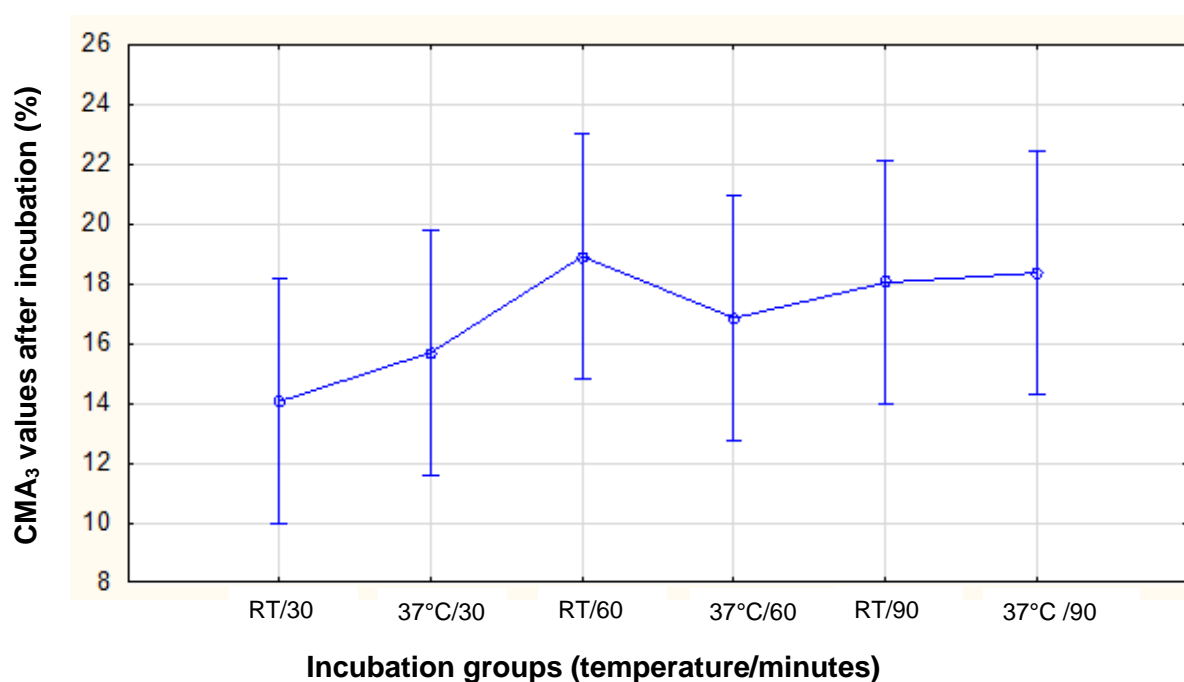


**Figure 3.4: The LS means of the motility after incubation of the processed semen sample (n=33) in 6 incubation groups (p< 0.05)**

### 3.2.1.2 CMA<sub>3</sub>– post-preparation

The effect of incubation time and temperature on sperm CMA<sub>3</sub> is presented in Table V and Figure 3.5 (n=33). The CMA<sub>3</sub> values are expressed as percentage positive (% DNA abnormal). The values ranged between 0 and 59 with 2 samples having uncertain abnormality (41-60%). This was seen in the 37°C/60 and the RT/90 groups.

CMA<sub>3</sub> values (%) remained relatively constant between the different incubation groups [RT/30: 14(9-18); 37°C/30: 14(6-20); RT/60: 17(8-28); 37°C/60: 15(9-20); RT/90: 12(9-25); 37°C/90: 15(8-25)] (Table V). It appeared that the RT/60 group had the highest CMA<sub>3</sub> score (therefore the worst chromatin packaging), but no statistical differences were observed between the groups (p=0.18) (Figure 3.5).

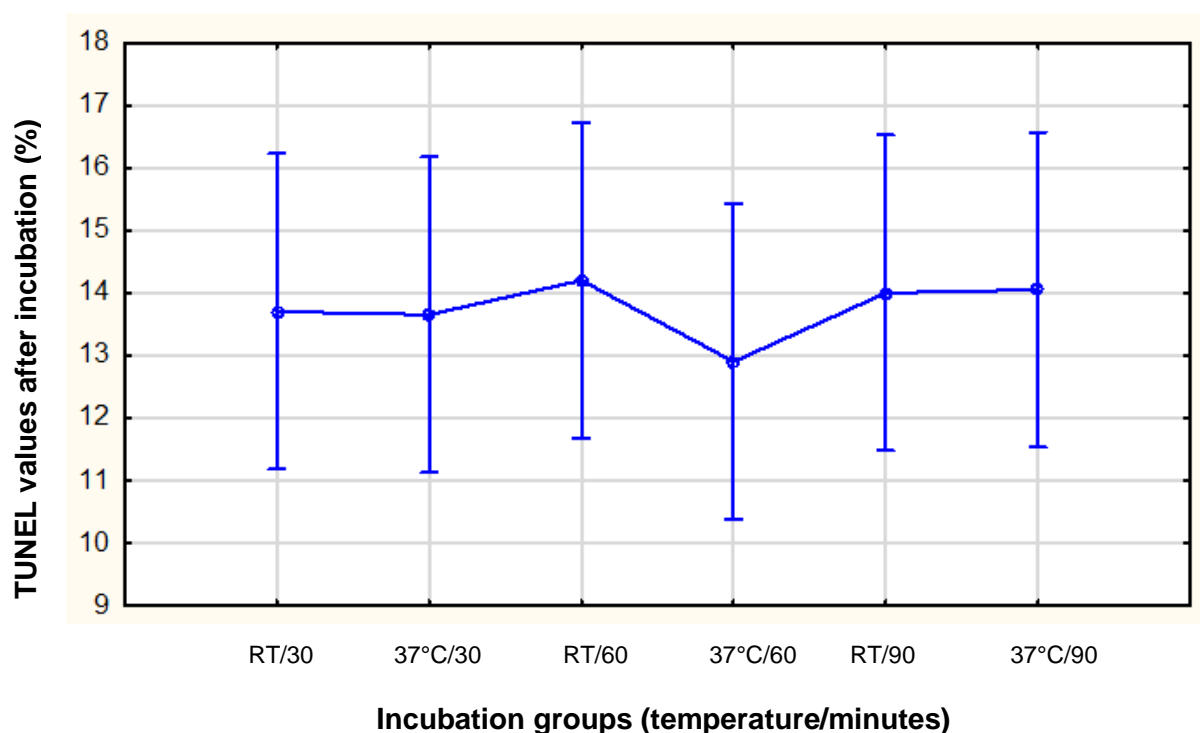


**Figure 3.5: The LS means of the CMA<sub>3</sub> values (%) after incubation of the processed semen sample (n=33) in 6 incubation groups (p>0.05)**

### 3.2.1.3 TUNEL– post-preparation

The effect of incubation time and temperature on sperm DNA fragmentation [TUNEL] is presented in Table V and Figure 3.6 (n=20). The TUNEL values are expressed as percentage positive (% DNA abnormal) sperm. The values ranged between 3 and 30 %, falling in the normal range ( $\leq 30$  % positive sperm cells)

The TUNEL values (%) between the groups remained stable [RT/30: 12.5(9-18); 37°C/30: 13(9-18); RT/60: 11(10-17); 37°C/60:15(10-16); RT/90: 16(9-20); 37°C/90: 15(10-17)] (Table V) and no statistical significant differences were found between the groups ( $p=0.95$ ).



**Figure 3.6: The LS means of the TUNEL values (%) after incubation of the processed semen sample (n=20) in 6 different incubation groups ( $p>0.05$ )**

### 3.2.1.4 TUNEL and CMA3 correlation – post-preparation

The fixed effect test, with a restricted maximum likelihood, determined that there is no relationship between TUNEL and CMA3 ( $p=0.89$ ).

### **3.2.2. Summary: Prospective Study 2 – post-preparation semen samples**

- Incubation time and temperature had a significant effect on the motility of the post-preparation sperm
  - The effect was most evident in the RT/90 group, where the motility was significantly lower compared to the other groups
- Incubation time and temperature did not have a significant effect on the CMA<sub>3</sub> and TUNEL results
- No association between TUNEL and CMA<sub>3</sub> was found

### **3.3 SECONDARY OBJECTIVE: THE EFFECT OF INCUBATION TIME ON THE ART OUTCOME – A RETROSPECTIVE STUDY**

The results of this retrospective study are summarized in Table VII – X and Figures 3.7-3.12. Descriptive data is presented in Table VII and Figure 3.7.

A total of 901 patient ART cycles (January 2010-December 2012) were included in this study – 212 IVF (23.45%) and 688 (76.11%) ICSI cycles. The mean female age was  $33.72 \pm 5.74$  years (range 19-48). An overall pregnancy rate of 35.94% was obtained in this cohort of patients. In 212 IVF cycles, 1423 MII oocytes were retrieved (6.74 oocytes per cycle). Of the total retrieved, 76.13% (1081/1423) were fertilized. In 688 ICSI cycles, 4987 MII oocytes were retrieved (7.21 oocytes/cycle). Of the total retrieved, 63.90% (3135/4987) were fertilized. As expected, the mean normal morphology and count (IVF vs. ICSI) was higher in the IVF group: normal morphology ( $10.67 \pm 3.51$  vs.  $6.04 \pm 3.73$  respectively) and count ( $35.98 \pm 18.64$  vs.  $13.51 \pm 14.54$  respectively). The mean sperm incubation time (time from final semen preparation to insemination/injection) of 70.99 and 186.19 minutes for IVF and ICSI respectively, was found to be significantly different ( $P < 0.001$ ) (Figure 3.7).

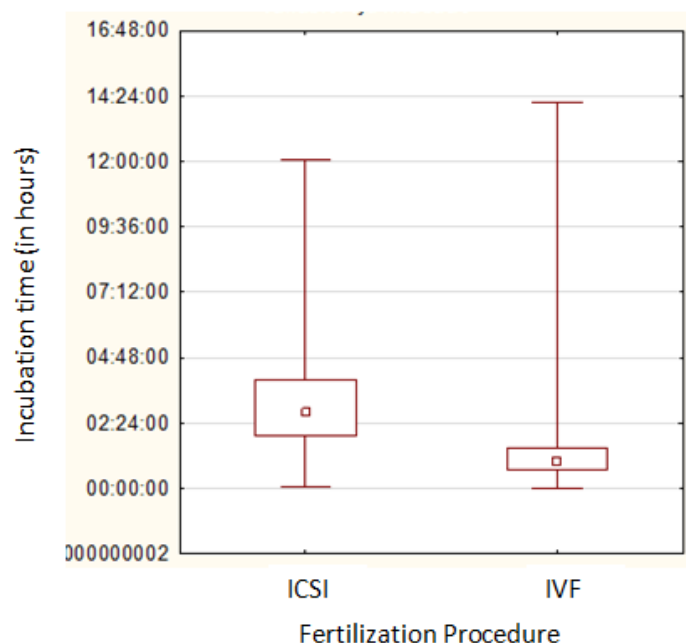
Pregnancy rate in this report is defined as a positive serum  $\beta$ hCG (IU/ml) ( $>10$  IU/ml).



**Table VII: Descriptive Statistics of the IVF and ICSI outcomes. Mean  $\pm$ SD values are displayed**

	Overall	IVF	ICSI
<b>Number of Patients</b>	904	212	688
<b>Mean Female Age (years)</b>	33.72 $\pm$ 5.74	33.50 $\pm$ 5.34	33.82 $\pm$ 5.83
<b>Sperm Count (<math>\times 10^6</math>/ml) after preparation</b>	18.80 $\pm$ 18.26	35.98 $\pm$ 18.64	13.51 $\pm$ 14.54
<b>Normal Morphology (n=731)<sup>b</sup></b>	7.35 $\pm$ 4.23	10.67 $\pm$ 3.51 (n=206)	6.04 $\pm$ 3.73 (n=521)
<b>Number of retrieved MII oocytes/cycle <sup>a</sup></b>	7.10 $\pm$ 4.09	6.74 $\pm$ 4.14	7.17 $\pm$ 4.02
<b>Mean Incubation Time (min)</b>	159.26 $\pm$ 105.12	70.99 $\pm$ 69.12	186.19 $\pm$ 90.72
<b>Minimum Incubation time (min)</b>	0	0	4.99
<b>Maximum Incubation Time (min)</b>	849	849	724
<b>Fertilization rate (%)</b>	66.79 $\pm$ 23.60	76.13 $\pm$ 23.20	63.90 $\pm$ 23.04
<b>Pregnancy Rate (%)</b>	35.94	31.10	36.44

<sup>a</sup> MII - Metaphase Two<sup>b</sup> Excluded donor sperm



**Figure 3.7: Box and Whisker plot depicting the significant different incubation times of processed semen samples in IVF (n=212) and ICSI (n=688) ( $p<0.01$ )**

Table VIII shows that the data (IVF and ICSI) was categorized in 5 groups according to sperm incubation time: group 1 with an incubation time of 1 hour or less, group 2 between 1 and 2 hours, group 3 between 2 and 3 hours and group 4 more than 4 hours. Statistically, the pregnancy rates between the different groups were found to be non-significant ( $P= 0.96$ ).

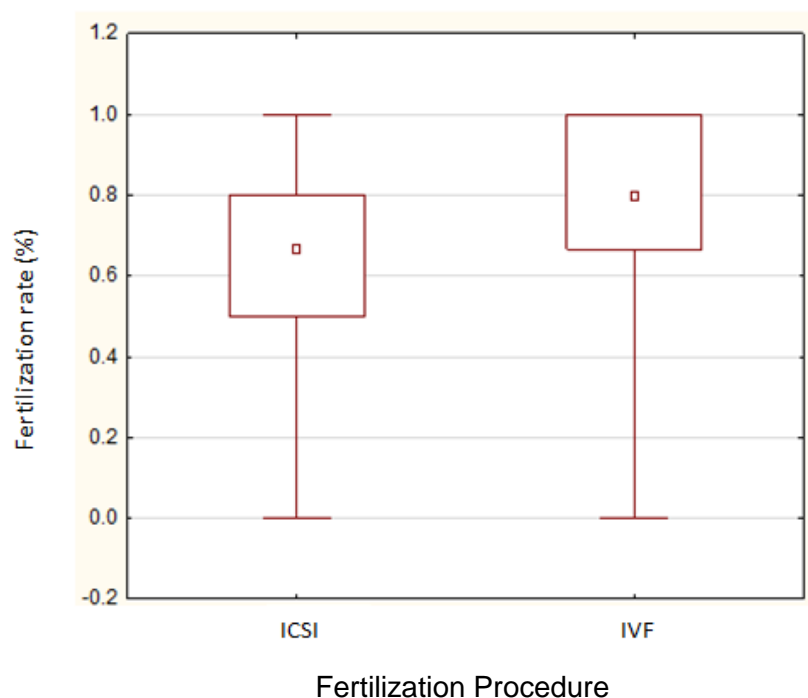
**Table VIII: The fertilization and pregnancy rates for the different time intervals of processed semen samples. Mean  $\pm$ SD values are displayed**

Incubation time	Group 1 < 1hour	Group 2 >1 and <2 hours	Group 3 >2 and < 3 hours	Group 4 >3 and < 4 hours	Group 4 >4 hours
Number of Patients	163	202	220	147	171
Mean Female Age (years)	32.69 $\pm$ 6.00	34.62 $\pm$ 5.29	32.83 $\pm$ 5.99	33.76 $\pm$ 5.46	34.74 $\pm$ 5.61
Fertilization rate (%)	71.95 $\pm$ 24.30	68.06 $\pm$ 25.60	66.60 $\pm$ 21.34	62.09 $\pm$ 21.87	64.57 $\pm$ 23.98
Pregnancy Rate (%)	33.13	26.82	26.24	22.15	24.56

### 3.3.1 Effect of Sperm Incubation Time, Post-Preparation, on Fertilization Rates in IVF and ICSI

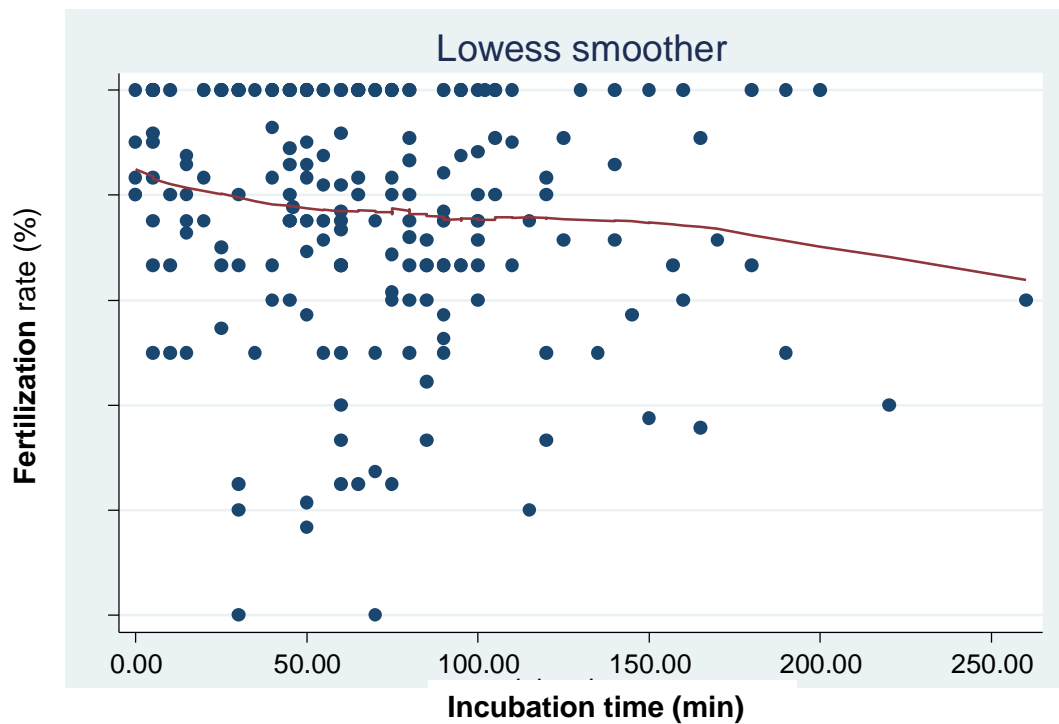
In this study group, female age was not significantly associated with fertilization rates (%) ( $P>0.05$ ).

In Figure 3.8, using a Box and Whisker plot, the difference in ICSI and IVF fertilization rates are presented. A Mann-Whitney U-test showed that there was a significant difference in fertilization rates between IVF and ICSI [ $76.13\pm23.20$  and  $63.90\pm23.04$ , respectively] ( $P<0.05$ ).



**Figure 3.8: Box and Whisker Plot depicting the significant difference in fertilization rates (%) in IVF (n=212) and ICSI (n=688) cycles ( $p<0.05$ )**

Sperm incubation time did not have an effect on the ICSI fertilization rates ( $p=0.28$ ), however a significant negative association ( $P=0.041$ ) was observed with the IVF fertilization rate (Figure 3.9). For every extra minute of sperm incubation in IVF cycles, the chance of fertilization decreases with .996 % ( $OR = -0.0037785 \times 1$ ) (95%CI: .992 to .999).



**Figure 3.9: Lowes Smoother Graph showing the significant negative association between incubation time and fertilization rates in IVF cycles (n=212) (P=0.041)**

### 3.3.2 Effect of Sperm Incubation Time, Post Preparation, on Embryo Quality in IVF and ICSI

Table IX shows the incidence of good quality embryos in the study cohort. The number of embryos on day 2, day 3 and day 5 were 4202, 3955 and 3014 respectively (Table IX). There were substantially more good quality embryos on day 2 compared to day 3 (68.47% vs. 50.95%) – On day 5 the percentage of good quality embryos was very low (1.89%). This is due to the strict criteria that define a good quality day 5 embryo. Embryos that did not form blastocyst on day 5 were also considered poor quality embryos.

**Table IX: Number and percentage of good quality embryos on day 2, day 3 and day 5**

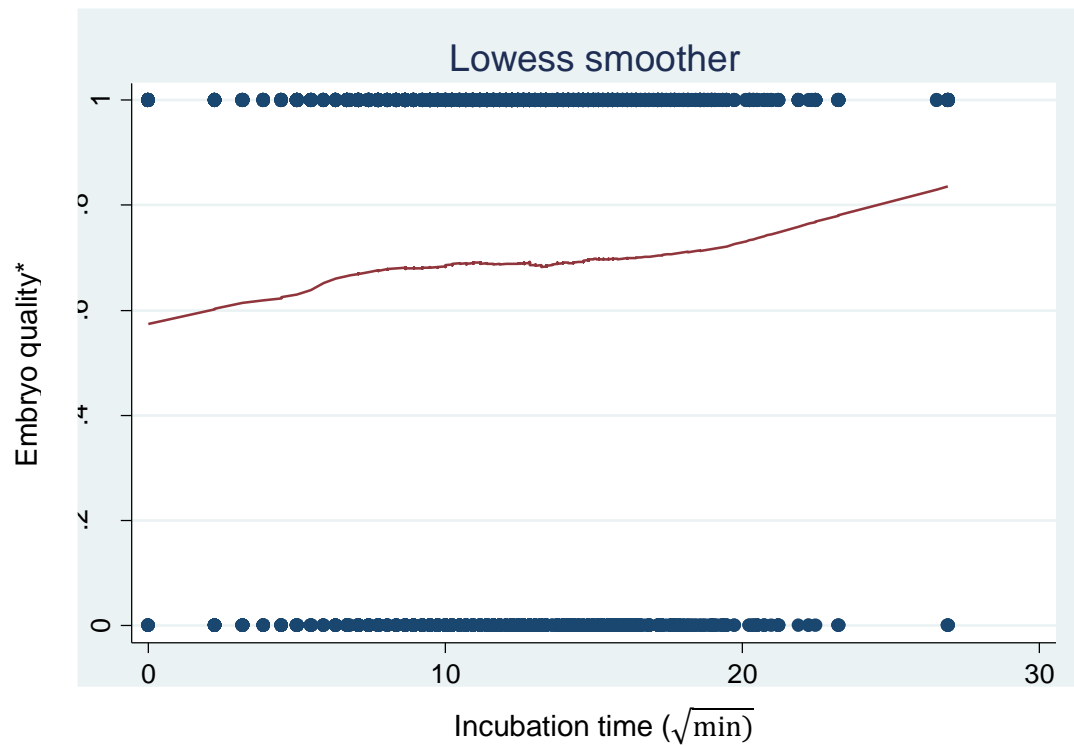
	Day 2	Day 3	Day 5
<b>Number of Embryos</b>	4202	3955	3014
<b>% Good Quality</b>	68.47	50.95	1.89

The binominal regression model showed that female age did not have a significant effect on embryo quality ( $P = 0.886$ ).

Figure 3.10 (IVF and ICSI) showed that the embryo quality of day 2 embryos increased as sperm incubation time increased and this was positively significant as assessed by the binominal regression model ( $p = 0.025$ ). For every 100 minute extra incubation time, the portion of good quality embryos increased by 5.4% (95% CI; 7% -10.1%) (Binominal regression model).

There was no association between the quality of day 3 embryos and incubation time ( $p = 0.93$ ; binominal regression model).

Due to low numbers of good quality embryos on day 5 no statistical analysis could be performed.



\*The 0 on the y-axis refers to poor quality and the 1 refers to good quality embryos.

**Figure 3.10: Lowess smoother plot showing the positive effect of incubation time on the embryo quality of day 2 embryos in IVF and ICSI cycles (n=904) (p=0.025)**

### 3.3.3 Effect of Sperm Incubation Time, Post-Preparation, on Pregnancy Rates in IVF and ICSI

Overall, there was a significant negative association between female age and pregnancy rates ( $P=0.002$ ) - Mann-Whitney signed rank test (Table X). The fertilization rate did not have an effect on pregnancy rates ( $P=0.272$ ).

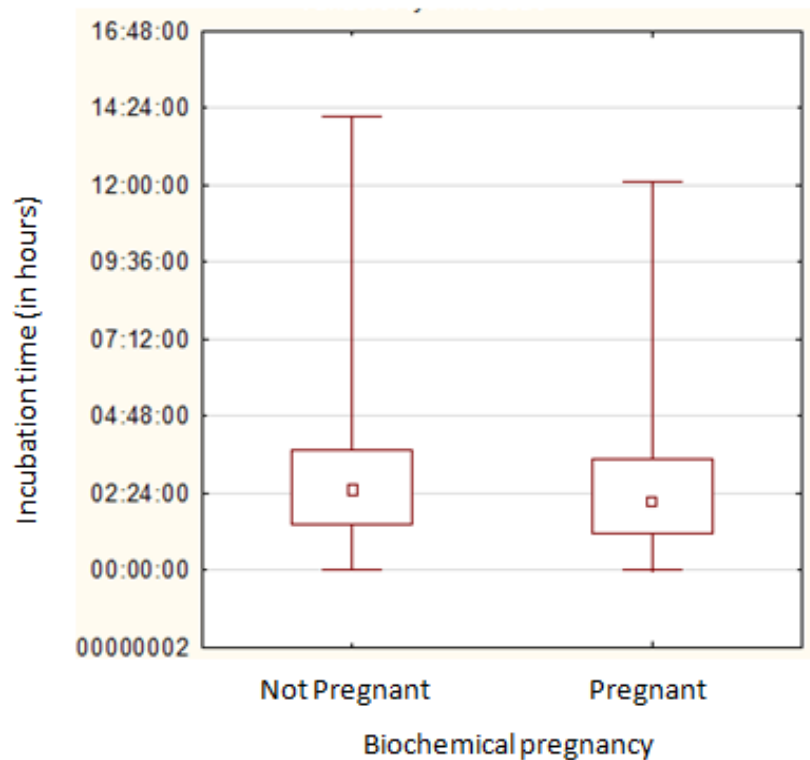
A significant difference in the post-preparation sperm incubation time was found between pregnant and non-pregnant women ( $P=0.046$ ) overall (IVF and ICSI cycles) (Table XI) with shorter sperm incubation time significantly associated with pregnancy. There were also significantly less MII oocytes retrieved/cycle in the non-pregnant group ( $P=0.001$ ).

**Table X: Different outcomes between pregnant and non-pregnant couples in IVF and ICSI. Mean  $\pm$ SD values are displayed**

	Pregnant	Not Pregnant	P
Number of patients	239	665	-
Average Age (SD)	32.66 $\pm$ 5.93	34.11 $\pm$ 5.62	0.002
Sperm Count ( $\times 10^6$ ) after preparation	18.79 $\pm$ 18.32	18.80 $\pm$ 18.26	0.931
Normal Morphology (n=731)	7.47 $\pm$ 4.29	7.31 $\pm$ 5.92	0.709
Number of retrieved oocytes MII/cycle	7.70 $\pm$ 4.23	6.88 $\pm$ 4.01	0.001
Mean Sperm Incubation Time (min)	152.64 $\pm$ 113.76	161.76 $\pm$ 101.72	0.046
Fertilization rate (%)	68.7 $\pm$ 20.3	66.10 $\pm$ 24.3	0.272

P value < 0.05000 is significant as measured by the Mann-Whitney signed rank test and indicated in red

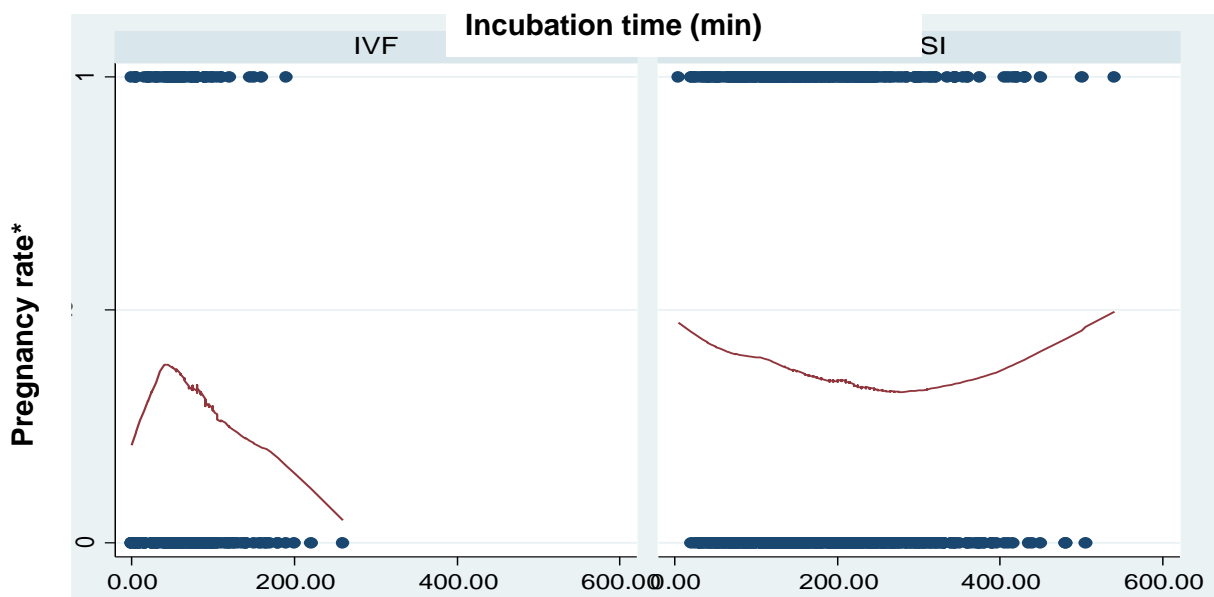
A Box and Whisker Plot also shows the significant increase in sperm incubation ( $161.76 \pm 101.72$  vs.  $152.64 \pm 113.76$ ) time (minutes) between the non-pregnant and the pregnant groups, retrospectively (Figure 3.11).



**Figure 3.11: Box and Whisker Plot depicting the significant different sperm incubation times between pregnant and non-pregnancy couples ( $p < 0.05$ ).**



When the ICSI and IVF groups were analysed separately, a Lowess smoother plot (Figure 3.12) showed that for IVF cycles, the number of pregnancies decreased with longer post-preparation sperm incubation times, while ICSI pregnancy outcome remained relatively constant. However, when the results were corrected for female age (logistic regressive test), the decrease in IVF pregnancy rate was not significant ( $p=0.154$ ). No relationship was found between pregnancy rates and incubation time in IVF and ICSI cycles, although the odds of falling pregnant were marginally in favour of ICSI ( $OR = .9987$  vs.  $.9957$ ).



\*The 0 on the y-axis refers to no pregnancy and the 1 refers to pregnancy,

**Figure 3.12: Lowess smoother graph showing the effect of incubation time on pregnancy rates in IVF (n=212) and ICSI (n=688) cycles**

### 3.3.4 Summary

- Post preparation sperm incubation times between IVF and ICSI differed significantly
- Age did not have an effect on fertilization rate or embryo quality
- Number of pregnancies decreased with female age
- Post-preparation sperm incubation time negatively and significantly affected fertilization rates in IVF (not in ICSI)
- Longer incubation times lead to a higher number of good quality day 2 embryos
- Longer post-preparation sperm incubation time was significantly associated with failure to fall pregnant in the overall study group (IVF and ICSI).
  - Although post-preparation sperm incubation time was significantly, negatively associated with specifically IVF pregnancy rates, correction for female age removed the significance
- Incubation time of sperm is significantly different between pregnant and non-pregnant couples

## CHAPTER 4: DISCUSSION AND CONCLUSION

Semen samples used in ART are collected at different times – before or after the ovum pick-up [OPU] procedure. During these incubation times, the sperm cells are exposed to factors that may inflict damage to the sperm structure and DNA integrity, impair its functional abilities, and subsequently lead to fertilization failure and poor ART outcome. Sperm DNA damage has been indicated in many aspects of poor ART outcome and there is a risk that spermatozoa carrying damaged DNA are being used clinically. The incubation temperature and time are two important factors that can potentially affect the sperm quality. No consensus has yet been reached on what the ideal conditions are for these parameters. It is therefore crucial that the time and temperature of incubation is standardized to optimize the ART outcome. Implementing standard procedures will facilitate best practices in the ART lab and cut-off incubation times and temperature for semen incubation should be available.

**The primary objective of this study is to determine the effect of different incubation times and temperatures on motility and the DNA profile of sperm cells. The CMA<sub>3</sub> assay was applied to determine the chromatin maturity and compaction and the TUNEL assay to assess the level of DNA fragmentation. The analysis was done on sperm samples randomly selected from male patients visiting a fertility clinic and adhering to the inclusion criteria set out in the Materials and Methods section.**

The sperm count remained constant in both the non-processed and processed incubation groups and no significant difference was found between the groups. Therefore, we can be assured that the samples were divided equally in terms of sperm count for the analysis.

The effect of incubation time and temperature was evident in the difference in motility of the sperm before and after preparation. For the **non-processed** sample incubation, sperm motility was higher when incubated at 37°C compared to incubation at RT. The motility declined over

the 20 minute time intervals. No difference in motility was found between 20 minutes and 40 minutes incubation at 37°C; similarly, there was no difference between 40 and 60 minutes at 37°C. There is a significant difference between 20 and 60 minutes at 37°C. It can therefore be concluded that to obtain a sample with a high percentage of motile sperm pre-preparation, the ideal time/temperature option is at 37°C for 20 minutes, but incubation for 40 minutes is not significantly different and also acceptable. Although statistical differences were found, it is important to note that clinically all motility values for all temperature/combinations were within acceptable ranges for ART (lowest average motility 54% and highest 57%). Similar to the non-processed group, a decrease in motility over time was also seen in the **processed** sample group. Higher temperatures (37°C) lead to a higher percentage of motile sperm. The difference in motility was most evident when the sample was incubated for 90 minutes at RT. It can therefore be concluded that to preserve motility, the sample should be incubated at RT or 37°C for 30 minutes after preparation. The sample can be incubated for up to 90 minutes at 37°C and should not be incubated for more than 30 minutes at RT. Although statistical significance was found, these values were again within acceptable ranges for ART (lowest average motility 79.78% and highest 84.18%)

Most studies agreed that sperm motility decreased in a time-dependent manner (Aitken *et al.*, 1996; Calamera *et al.*, 2001; Matsuura *et al.*, 2010); however, studies also found no difference in motility after incubation times (Lachaud *et al.*, 2004; Petrella *et al.*, 2003; Jackson *et al.*, 2010). Studies regarding the effect of incubation temperature on motility report contradictory results. Results similar to our study were found by Esfandiari *et al.* (2002) concerning the optimum temperature for motility when incubating the non-processed sample is 37°C. Most studies disagreed and concluded RT is the ideal temperature at which samples should be incubated to preserve motility function (Calamera *et al.*, 2001; Schuffner *et al.*, 2002; Lachaud *et al.*, 2004). Matsuura *et al.* (2010), found that prolonged incubation (time intervals 0, 3, 5, 24 hours) before preparation at 37°C reduced the motility of the sperm more rapidly compared to RT. Similarly, Thijssen *et al.*, (2013) found a significantly longer preservation of sperm motility

post-preparation when samples were incubated at RT. Marin-Briggiler *et al.*, (2002) found similar percentages of motile sperm cells at RT and 37°C.

Sperm motility is temperature-dependent and assessment should be carried out as close to body temperature as possible. Prolonged incubation at 37°C may reduce sperm motility (Calamera *et al.*, 2001; Lachaud *et al.*, 2004). The effect of lower temperature on sperm function preservation may be due to the sperm cells residing in a resting state at the lower temperature and thus preservation of their energy (Thijssen *et al.*, 2013). The good preservation of post-preparation motility at 37°C established in our study (even at 90 minutes incubation) is therefore expected. One can argue that lower temperatures could act to reduce the metabolic activity of the sperm cells and sustain their viability *in vitro*. The decrease in motility over time before and after the sample was processed could be attributed to increased ROS levels; ROS has been shown to affect motility (Aitken and Clarkson, 1997). This decline may be caused by a depletion in adenosine triphosphate (ATP), which affects the sperm cells' axonemes. ROS can also affect the mitochondrial function. Hydrogen peroxide produced by ROS can at low quantities also affect sperm cell function (Esfandiari *et al.*, 2002). In the non-processed group, the seminal plasma contains components (leukocytes, senescent sperm cells) that can increase the ROS levels and in the processed semen sample, high ROS levels may be present due to the manner in which the sample was prepared. Centrifugation can damage the sperm cells directly by the mechanical process or indirectly through the adverse effect caused by ROS (Shekarriz *et al.*, 1995) ROS affects the membrane integrity of the sperm cell, which is closely correlated with motility (Aitken and Clarkson, 1997). It also depletes the ATP store, which in turn influences enzyme function (Aitken and Clarkson, 1997). An inverse relationship has been described between the amount of ROS detected and the percentage motile sperm (Iwasaki and Gangnon, 1992).

CMA<sub>3</sub> detects protamine deficiency in loosely packed chromatin. Increased CMA<sub>3</sub> values (less mature sperm) were seen in the **non-processed** group over longer incubation time periods and higher temperatures (37°C), however the increase was not significant. In the **processed**

group, CMA<sub>3</sub> values increased over time at 37°C, but it was found that the temperature did not play a role post-preparation. Even though no significant effect was found, our results suggested that for CMA<sub>3</sub> pre-preparation incubation time should be kept at a minimum and at a lower temperature. To our knowledge, no studies investigating the effect of semen incubation time and temperature on the CMA<sub>3</sub> values have been published. It was found that the sperm cells' resistance to heat DNA denaturation is dependent on the maturity of the sperm (Eggert-Kruse *et al.*, 1996); therefore, in immature sperm the higher temperatures will have a negative effect on the DNA.

DNA fragmentation was measured using the TUNEL assay. In the **non-processed** group, there was no association observed between TUNEL outcome and incubation time and temperature. Higher TUNEL values were found in general after longer incubation periods (> 40 minutes) and at RT. The DNA fragmentation levels in the **processed** group remained stable. It can therefore be concluded that time and temperature does not have an effect on the DNA fragmentation, incubation for prolonged periods in the non-processed sample at 37°C can be suggested.

Our findings on incubation time and DNA fragmentation can be compared with Matsuura *et al.*, 2010, who found a time-dependent increase in the DFI values (as measured by SCSA). They did not find that samples should be incubated at 37°C; in their study 37°C led to an increase in the DFI value (as measured by SCSA) and RT was the recommended incubation temperature. Dalzell *et al.*, 2004 also showed that that incubation of sperm at 37°C increases sperm DNA fragmentation (Dalzell *et al.*, 2004). This was, however, in a 4 our time-span. Our findings concerning incubation time were similar to that of Toro *et al.* (2009) where a significant increase in DNA fragmentation in non-processed incubation intervals was found. Likewise, Muratori *et al.*, (2003) and Rougier *et al.* (2013) confirmed that DNA fragmentation increases spontaneously over time. Nabi *et al.* (2014) suggested that samples should not be incubated after preparation for more than 2 hours (37°C) to minimize the amount of DNA damage (as measured by SCD).

Mammalian sperm are very sensitive to excess heat and the results showing the increase in DNA fragmentation at ambient temperatures is unexpected. Elevated temperature leads to DNA denaturation; while incubation at lower temperatures should be more appropriate to conserve sperm quality and reduce DNase activity. To explain the DNA fragmentation observed over longer incubation time, the involvement of a sperm endonuclease activity can be postulated, as it occurs in somatic cells (Wyllie *et al*, 1980; Ishizaki *et al.*, 1995). Another possible cause of spontaneous DNA fragmentation is ROS production by sperm (Muratori *et al.*, 2003).

No correlation was found between the different sperm tests (TUNEL and CMA<sub>3</sub>) for non-processed and processed sperm samples. Subsequently, no relationship between DNA compaction and DNA fragmentation could be described. This differs from a study which found that the amount of protamine deficiency in loosely packed chromatin is correlated to the extent of nicked DNA (Manicardi *et al.*, 1995).

**As a secondary objective, we retrospectively investigated the effect of sperm incubation time of the processed semen sample on ART outcome (January 2010 - December 2012) (fertilization rates, embryo quality and pregnancy rates).**

There was a significant association between the ART procedure (IVF/ICSI) and the fertilization rates. IVF cycles had a higher fertilization rate compared to ICSI cycles. Longer incubation times had a significant negative effect on the fertilization rates in the IVF group; this association was valid over a linear scale. Odds ratios analysis showed that the odds of falling pregnant are in favour (20% more likely) in a female where sperm cells were incubated for a one hour shorter sperm incubation time. Sperm incubation times of the processed sample did not affect the ICSI fertilization rates. There was also no association between female age and fertilization rates. Therefore, it can be concluded that shorter sperm incubation times in IVF are more beneficial to the fertilization rates.

Low fertilization rates in IVF are not unexpected (Hershlag *et al.*, 2002; Jaroudi *et al.*, 2003; Hwang *et al.*, 2005; Foong *et al.*, 2006). Often sperm abnormalities are not obvious even if the semen analysis is normal and the patient is accepted for IVF. At the Aevitas Fertility Clinic sperm DNA tests are not routinely done and patients with compromised sperm DNA can be included in an IVF cycle. This may result in poorer IVF fertilization. The lower fertilization results in IVF may also hypothetically be related to an excessive spontaneous acrosome reaction in the culture medium, especially after a long incubation period. The acrosome reaction has been shown to be time-dependent (Mansour *et al.*, 2008). In IVF, sperm cells need to bind to the zona pellucida with its acrosome intact and this binding stimulates the sperm cell to undergo the acrosome reaction (Kopf and Gerton, 1991), which is a prerequisite for fertilization. In order for the acrosome reaction to occur, the sperm cells need to undergo capacitation and this is induced by the synthetic media and has been shown to be temperature-dependent (Visconti and Kopf, 1998; Marin-Briggler *et al.* 2002). Temperature changes have been shown to affect membrane lipid diffusibility and peroxidation, as well as antigen distribution on the sperm cell plasma membrane (Villarroya and Scholler, 1987). Sperm however also need to capacitate for normal fertilization to take place – so the incubation time and temperature of post-preparation sperm samples for IVF should be carefully monitored.

With ICSI, all barriers to sperm penetration are bypassed and sperm selection is performed subjectively by the micro-manipulator. Acrosome-reacted sperm may be a prerequisite for ICSI to avoid irregular sperm decondensation, however Sathananthan *et al.* (1997) found that the acrosome reaction can occur in the oocyte and lead to normal fertilization, thus it is not a prerequisite before ICSI. The highest fertilization rate for ICSI was reported when the sample was incubated for 3 hours (Mansour *et al.*, 2008; Patrat *et al.*, 2012). Another possible explanation that can affect the fertilization rates is the DNA damage to the sperm. Studies are contradictory on the effect of DNA fragmentation on fertilization. Benchaib *et al.* (2003) found that DNA fragmentation influenced fertilization rate in ICSI cycles and not in IVF. From this



result we can conclude that sperm DNA fragmentation does not play a role in IVF. It has also been suggested that sperm cells with fragmented DNA can fertilize oocytes with the same efficiency as sperm cells without DNA fragmentation (Twigg *et al.*, 1998; Tomlinson *et al.*, 2001; Morris *et al.*, 2002).

Prolonged incubation can compromise the fertilizing ability of the sperm cells due to various factors and processes, including 1) depletion of energy sources in the sperm-washing medium, 2) premature capacitation (Yavas and Selub, 2004) and 3) DNA fragmentation. Marin-Briggiler *et al.* (2002) found that capacitation is inhibited at 20°C and speculated that a temperature of 20°C blocks the removal of cholesterol and subsequently inhibits protein tyrosine phosphorylation (Marin-Briggiler *et al.*, 2002). If the sample is incubated for too long, capacitation and subsequently the acrosome reaction occurs prematurely and the acrosome-reacted sperm cells are unable to bind to the zona pellucida. This will negatively affect the sperm cells' ability to fertilize the oocyte.

In our study, longer sperm incubation times had a positive effect on the quality of day 2 embryos (in IVF and ICSI). There was no association between incubation time and quality of day 3 embryos, and day 5 embryos were excluded from the analysis due to the low cohort of good quality embryos. No other studies that observed the effect of sperm incubation time on embryo quality could be found in the literature.

The first steps of embryo development are under the influence of the maternal transcripts, thus the paternal expression would normally only start at the 6-8 cell stage. If critical genes are damaged when the paternal genome is activated at day 3 embryo development failure is likely to occur and this can explain the lower percentage of good quality embryos found on day 3 compared to day 2.

Several studies on the effect of sperm DNA damage on embryo quality have been published. Studies show that there was no consistent relationship between sperm DNA damage and embryo quality in IVF cycles. Some studies did not identify any adverse effect of sperm DNA

damage on embryo quality (Lopes *et al.*, 1998; Tomlinson *et al.*, 2001; Larson-Cook *et al.*, 2003; Benchaib *et al.*, 2003; Huang *et al.*, 2005) while others reported a negative correlation (Morris *et al.*, 2002; Seli *et al.*, 2004; Virro *et al.*, 2004; Zini *et al.*, 2005). Shoukir *et al.* (1998) reported that abnormal morphology of sperm cells may have a negative paternal effect on pre-implantation embryo development. They suggested that defective protamine packaging in sperm DNA and their incorrect replacement by histones during fertilization may create problems such as asynchronous cleavage and delays in the cell cycle after fertilization, thereby lowering blastocyst formation.

Even though longer incubation times of the processed semen sample led to decreased fertilization rates, but better embryo quality, one cannot assume that it is just the contribution of the sperm cells. During the incubation of the processed samples oocytes undergo nuclear and cytoplasmic changes that play a crucial role in the quality and maturity of the oocyte and its ability to undergo fertilization and development of the embryos (Zenzes *et al.*, 1985). In IVF, oocyte fusion does not solely depend on the maturity (acrosome-reacted) of the sperm cells; a mature plasma membrane of the oocyte is also necessary to achieve fusion (Yanagida *et al.*, 1998). Cytoplasmic immaturity can also be related to polyspermy, which also reduces the fertilization rate (Ho *et al.*, 2003). Thus fertilization failure could be caused by either the sperm cell or the oocyte.

As expected, the **pregnancy rates decreased with female age**. ICSI cycles resulted in higher pregnancy rates compared to IVF; however, there was no statistically significant association between pregnancy rates and the ART procedure. Cycles that resulted in a pregnancy had a modest, but significant lower sperm incubation times. IVF cycles appeared to be significantly affected by the longer sperm incubation times but this was however found to be non-significant when female age was also considered as a factor in statistical analysis. Sperm incubation time did not affect the ICSI pregnancy rates at all. It can therefore be concluded that shorter incubation times is favourable, however we cannot ascertain if this is applicable in both IVF and ICSI.

Yavas *et al.* (2004) compared incubation times of the processed sample and IUI outcome with different stimulation protocols (clomiphene citrate and hMG). In contrast to our study, the incubation time did not affect pregnancy rates in the clomiphene citrate group. However, in the hMG group, the incubation time was shorter in pregnant vs. non-pregnant women. This supports our finding that pregnancy is enhanced by shorter sperm incubation time intervals. A possible limitation to our study is that we did not differentiate between different stimulation protocols in the analysis. With longer sperm incubation time, there was an increase in DNA fragmentation and that may lead to the significantly reduced pregnancy rates observed in our study. Several studies have evaluated DNA structure and integrity and reported a negative effect of sperm DNA damage on pregnancy rates (Tesarik, 2004). Nabi *et al.* (2014) suggested that a decrease in pregnancy rate could be due to the detrimental effect of incubation at 37°C on sperm DNA.

In general prolonged time intervals and elevated temperatures before and after the sample was processed had a negative effect on the motility of sperm cells. Although not significant, the DNA fragmentation and abnormal compaction was increased when samples were incubated for prolonged time intervals. The increased DNA fragmentation levels found at longer incubation times and temperatures is important as an increase in DNA fragmentation can lead to decreased fertilization (Simon *et al.*, 2010), affect the pre-implantation embryo development (Nasr-Eshafani *et al.*, 2005) and result in poor pregnancy outcome (Benchaiib *et al.*, 2003). The fertilization rates were affected by sperm incubation time in IVF cycles, but no effect was seen in ICSI cycles. Prolonged sperm incubation time was beneficial for the quality of day 2 embryos. Shorter sperm incubation time intervals were found in cycles that achieved pregnancy.

To conclude: Shorter sperm incubation time of non-processed and processed semen samples had the most beneficial effect on the motility of the sperm. In contrast with our hypothesis, we found that higher incubation temperatures were advantageous to the motility and also the DNA

fragmentation levels. It is therefore vital that the incubation time and temperature should be monitored.

Taking the results into consideration, we would recommend that:

- Incubate non-processed semen samples for both ICSI and IVF at RT or 37°C for no longer than 40 minutes
- After the sample has been processed, incubate IVF samples at RT or 37°C for no longer than 60 minutes (RT may be better)
- After the sample has been processed incubate ICSI samples at RT or 37°C for no longer than 60 minutes (RT may be better) – according to tested semen parameters
  - The in vitro results did not show an effect of incubation time on pregnancy rates – therefore a longer incubation time may be preferable (acrosome reaction).

Shortcomings of our study include small sample size (in TUNEL); longer time intervals should be assessed; leukocyte count should be taken into consideration, the DNA tests (TUNEL and CMA<sub>3</sub>) and semen parameters assessments are subjective; and inter-technician confirmation should be performed. In future, the effect of the oocyte pre-incubation time should also be considered in pinpointing the ideal collection and incubation time for semen samples.

## BIBLIOGRAPHY

Abou-haila A and Tulsiani DRP. Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Arch Bioch Biophys* 2009; 485: 72-81.

Agarwal A and Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update* 2003; 9: 331–345.

Agarwal A and Allamaneni SS. Sperm DNA damage assessment: a test whose time has come. *Fertil Steril* 2005; 84: 850–853.

Agarwal A, Saleh RA and Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 2003; 79: 829-843.

Ahmadi A and Ng SC. Fertilizing ability of DNA damaged spermatozoa. *J Exp Zool* 1999; 284: 696-704.

Aitken RJ. Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev* 1995; 7: 659-668

Aitken RJ and Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Androl* 1988; 2: 367-376.

Aitken RJ and Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 1997; 81: 459-469

Aitken JR and Baker MA. Reactive oxygen species generation by human spermatozoa: a continuing enigma. *Int J Androl* 2002; 25: 191-194.

Aitken JR and Baker MA. Oxidative stress, sperm survival and fertility control. *Mol Cell Endocrin* 2006; 250: 66-69.

Aitken RJ, Clarkson JS and Baker HWG. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in case of oligospermia. *J Androl* 1989; 10: 214.

Aitken RJ, Buckingham DW, West K, Wu FC, Zikopoulos K and Richardson DW. Differential contribution of leukocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. *J Reprod Fertil* 1992; 9: 367-376.

Aitken RJ, Buckingham DW, Brindle J, Gomez E, Baker HWG and Irvine DS. Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. *Hum Reprod* 1995; 10: 2061-2071.

Aitken RJ, Allan IW, Irvine DS and Macnamee M. Andrology: Studies on the development of diluents for the transportation and storage of human semen at ambient temperature. *Hum Rep* 1996; 11: 2186-2196.

Apedaile AE, Garrett C, Liu DY, Clarke GN, Johnston SA and Baker HWG. Flow cytometry and microscopic acridine orange test: relationship with standard semen analysis. *Reprod Biomed Online* 2004; 8: 398-407.

Appell RA and Evans PR. The effect of temperature on sperm motility and viability. *Fertil Steril* 1977; 28: 1329-1332.

Appell RA, Evans PR and Blandy JP. The effect of temperature on the motility and viability of sperm. *Br J Urol* 1977; 49: 751-756.

Bahadur G. Fertility issues for cancer patients. *Mol Cell Endocrinol* 2000; 169: 117-122.

Barroso G, Morshedi M and Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 2000; 15: 1338-1344.

Begley AJ and Quinn P. Decapacitation factors in semen. *Clin Reprod Fertil* 1982; 1: 167-175.

Benchai M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H, et al. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod* 2003; 18: 1023–1028.

Birkhead TR and Montgomerie R. Three centuries of sperm research. In: Sperm Biology: an evolutionary perspective. Birkhead TR, Fosken DJ, Pitnick SS (Eds). Elsevier, Oxford, UK. 2009. Pp. 1-36.

Bizzaro D, Manicardi GC and Bianchi PG. In situ competition between protamine and fluorochromes for sperm DNA. *Mol Hum Reprod* 1998; 4: 127-132.

Bjorndahl L. Basic semen analysis and laboratory quality for technicians. In: Clinical Andrology: EAU/ESAU Course Guidelines. Bjorndahl L, Giwercman A, Tournaye H and Weidner W (eds.). CRC Press, Florida, USA. 2010. P.32.

Bonde JPE, Ernst E, Jensen TK, Hjollund NH, Kolstad H, Scheike T, Giwercman A, Skakkebaek NE, Henriksen TB and Olsen J. Relationship between semen quality and fertility: a population-based study of 430 first-pregnancy planners. *Lancet* 1998; 352: 1172-1177.

Boomsma CM, Heineman MJ, Cohlen BJ and Farquhar C. Sperm preparation techniques for intrauterine insemination. *Cochrane Database Syst Rev* 2004; 3: CD4004507.

Bungum M, Humaidan P, Spano M, Jepson K, Bungum L and Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod* 2004; 19: 1401–1408.

Calafell JM, Badenas J and Egozcue J. Premature chromosome condensation as a sign of oocyte immaturity. *Hum Reprod* 1991; 6: 1017-1021.

Calamera JC, Fernandez PJ, Buffone MG, Acosta AA and Doncel GF. Effects of long-term incubation of human spermatozoa: functional parameters and catalase effect. *Andrologia* 2001; 33: 79-86.

Carrell DT, Emery BR and Hammoud S. Altered protamine expression and diminished spermatogenesis: what is the link? *Hum Reprod Update* 2007; 13: 313-327.

Cathcart R, Schwieters E, Saul RL and Ames BN. Thymine glycol and thymidine glycol in human and rat urine – a possible assay for oxidative DNA damage. *PNAS USA* 1984; 81: 5633-5637.

Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, Xhu Z, Schultz RM, Hecht NB and Eddy EM. Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. *Biol Reprod* 2003; 69: 211-217.

Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ and Carrell DT. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *J Androl* 2006; 27: 53–59.

Clarke GN. A.R.T and history, 1678-1978. *Hum Reprod* 2006; 21: 1645-1650.

Cohen CB. The moral sign of early human embryos in religious thought. In: *Renewing the stuff of life: stem cells, ethics and public policy*. Cohen CB (ed). Oxford University Press, New York, USA. 2007. P.100.

Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer MP and Wiemer K. Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. *Hum Reprod* 1990; 5: 7-13

Cross NL. Role of cholesterol in sperm capacitation. *Biol Rep* 1998; 59: 7-11.

Dalzell LH, McVicar CM, McClure N, Lutoon D and Lewis SE. Effects of short and long incubation on DNA fragmentation of testicular sperm. *Fertil Steril* 2004; 82: 1443-1445.

De Cássia da Silveira e Sá. Sperm capacitation: physiology, molecular mechanisms and relevance for fertilization. In: *Human Spermatozoa: Maturation, capacitation and abnormalities*. Lejeune T and Delvaux (Eds). Nova Science Publishers, New York, USA. 2010. Pp. 87-108.

De Paula TS, Bertolla RP, Spaine DM, Cunha MA, Schor N, Cedenho AP. Effect of cryopreservation on sperm apoptotic deoxyribonucleic acid fragmentation in patients with oligozoospermia. *Fertil Steril* 2006; 86: 597-600.

Di Santo M, Tarozzi N, Nadalini M and Borini A. Human sperm cryopreservation: Update on techniques, effect on DNA integrity and implications for ART. *Adv Urol* 2012; 2012: ID854837.



Donnelly ET, Steele EK, McClure N and Lewis SEM. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. *Hum Reprod* 2001; 16: 1191-1199.

Dozortev D, Rybouchkin A and De Sutter P. Human oocyte activation following intracytoplasmic sperm injection: the role of sperm cell. *Hum Reprod* 1995; 10: 403-407.

Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum Reprod* 2002; 17: 3122–3128.

Edwards RG. The history of assisted human conception with special reference to endocrinology. *Exp Clin Endocrinol Diabetes* 1996; 104: 183-204.

Eliasson R. Semen Analysis. *Environ Health Persp* 1978; 24: 81-85.

Eggert-Kruse W, Rohr G, Kerbel H, Schwalbach B, Demirakca T, Klinga K, Tilgen W and Runnebaum B. The acridine orange test: a clinically relevant screening method for sperm quality during infertility investigation? *Hum Reprod* 1996; 11:784-789.

Eppig JJ, Schultz RM, O'Brien M and Chesnel F. Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. *Develop Biol* 1994; 164: 1-9.

Erenpreiss J, Spano M, Erenpreiss J, Bungum M, Giwercman A. Sperm chromatin structure and male fertility: biological and clinical aspects. *Asian J Androl* 2006; 8: 11-29.

Esfandiari N, Saleh RA, Blaut AP, Sharma RK, Nelson DR, Thomas AJ, Falcone T and Agarwal A. Effects of temperature on sperm motion characteristics and reactive oxygen-species. *Int J Fertil* 2002; 47: 227-233.

Esterhuizen AD, Franken DR, Lourens JGH, Prinsloo E and Van Rooyen LH. Sperm chromatin packaging as an indicator of in vitro fertilization rate. *Hum Reprod* 2000; 15: 657-661

Evenson DP and Wixon R. Clinical aspects of sperm DNA fragmentation and detection and male infertility. *Theriogenology* 2006; 65: 979-991.

Faque P, Lehert P, Lamotte M, Bettahar-Lebugle K, Bailly A, Diligent C, Cledat M, Pierrot P, Guenedal ML and Sagot P. Clinical success of intrauterine insemination cycles is affected by the sperm preparation time. *Fertil Steril* 2014; 101: 1618-1623.

Fernández JL, Muriel L, Goyanes V, Segrelles E, Gosálvez J, Enciso M, LaFromboise M and De Jong C. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* 2005; 84: 833-842.

Flint M, Agarwal A and du Plessis SS. Leukocytospermia and oxidative stress. In: Studies on men's health and fertility. Agarwal A, Aitken RJ and Alvarez JG (Eds). Springer, New York, USA. 2012. Pp. 517-530.

Foong SC, Fleetham JA, O'Keane JA, Scott SG, Tough SC and Greene CA. A prospective randomized trial of convention in vitro fertilization versus intracytoplasmic sperm injection in unexplained infertility. *J Assist Reprod Gen* 2006; 23: 137-140.

Franklin S and Roberts C (eds). What is PGS? In: Born and made, an ethnography of preimplantation genetic diagnosis. Princeton University Press, New Jersey UK. 2006. Pp.52-54.

Fukuda A, Roudebush WE and Thatcher SS. Influences of in vitro oocyte aging on microfertilization in the mouse with reference to zona hardening. *J Assist Reprod Genet* 1992; 9: 378-383.

Gandini L, Lombardo F, Paoli D, Caruso F, Eleuteri P and Leter G. Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. *Hum Reprod* 2004; 19: 1409-1417.

Gasca S, Pellestor F, Assou S, Loup V, Anahory T, Dechaud H, de Vos J and Hamamah S. Identifying new human oocyte marker genes: a microarray approach. *Reprod Biomed Online* 2007; 14: 175-183.

Gatewood JM, Cook GR and Balhorn R. Sequence-specific packaging of DNA in human sperm chromatin. *Science* 1987; 236: 962-964.

Hammadeh ME, Stieber M, Haidl G and Schmidt W. Association between sperm cell chromatin condensation, morphology based on strict criteria and fertilization, cleavage and pregnancy rates in IVF programme. *Andrologia* 1998; 30: 29-35.

Hammadeh ME, Askari AS and Georg T. Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. *Int J Androl* 1999; 22: 155-162.

Hammadeh ME, Strehler E, Zeginiadou T, Rosenbaum P and Schmidt W. Chromatin decondensation of human sperm in vitro and its relation to fertilization rate after ICSI. *Arch Androl* 2001; 47: 83-87.

Hamoir, G. The discovery of meiosis by E van Beneden, a breakthrough in the morphological phase of heredity. *Int J Dev Biol* 1992; 36: 9-15.

Handyside AH, Pattinson JK, Penketh RJ, Delhanty JD, Winston RM and Tuddenham EG. Biopsy of human preimplantation embryos and sexing by DNA amplification. *Lancet* 1989; 18: 347-9

Hansen M, Kurinczuk JJ, Bower C and Webb S. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. *N Engl J Med* 2002; 346: 725–30.

Harrison RAP and Gadella BM. Membrane changes during capacitation with special reference to lipid architecture. In: Human Sperm acrosome reaction. Fenichel P and Parinaud J (Eds). John Libbey Eurotext, Montrouge/Paris, France.1995. p. 45-65.

Henkel R. Detection of DNA damage in sperm. In: Oehninger S, Kruger TF, editors. Male Infertility. Diagnosis and Treatment. Oxon, UK: Informa Healthcare; 2007. p. 225-238.

Henkel R and Schill WB. Sperm preparation for ART. *Reprod Bio Endo* 2003; 1: 1-22.

Henkel R, Kierspel E, Hajimohammad M, Stalf T, Hoogendijk C and Mehnert C. DNA fragmentation of spermatozoa and assisted reproduction technology. *Reprod Biomed Online* 2003; 7:477-484.

Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Gips H, Schill WB and Kruger TF. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* 2004; 81: 965-972.

Hershlag A, Paine T, Kvapil G, Feng H and Napolitano B. In vitro fertilization-intracytoplasmic sperm injection split: an insemination method to prevent fertilization failure. *Fertil Steril* 2002; 77(2): 229-232.

Ho JYP, Chen MJ, Yi YC, Guu HF and Ho ESH. The effect of preincubation period of oocytes on nuclear maturity, fertilization rate, embryo quality, and pregnancy outcome in IVF and ICSI. *J Assist Reprod Genet* 2003; 20: 358-364.

Hoogendijk CF and Henkel R. The pathophysiology and genetics of human male reproduction. In: Male Infertility. Diagnosis and Treatment. Oehninger S, Kruger TF (Eds). Informa Healthcare, Oxon, UK. 2007. p. 35-48.

Host E, Lindenberg S and Smidt-Jensen S. The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. *Acta Obstet Gynecol Scand* 2000; 79: 559-563.

Huang CC, Lin DP, Tsao HM, Cheng TC, Liu CH and Lee MS. Sperm DNA fragmentation negatively correlates with velocity and fertilization rates but might not affect pregnancy rates. *Fertil Steril* 2005; 84: 130–140

Hwang JL, Seow KM, Lin YH, Hsieh BC, Huang LW, Chen HJ, Huang SC, Chen CY, Chen PH and Tzeng CR. IVF versus ICSI in sibling oocytes from patients with polycystic ovarian syndrome: a randomized controlled trial. *Hum Rep* 2005; 20: 1261-1265.

Ickowicz D, Finkelstein M and Breitbart H. Mechanisms of sperm capacitation and the acrosome reaction: role of protein kinases. *Asian J Androl* 2012; 14: 816-812.

Isachenko E, Isachenko V and Katkov II. DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. *Hum Reprod* 2004; 19: 932-939.

Ishizaki Y, Cheng L, Mudge AQ and Raff MC. Programmed cell death by default in embryonic cells, fibroblasts, and cancer cells. *Mol Biol Cell* 1995; 6: 1443-1458.

Iwasaki A and Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992; 57: 409-416.

Jackson RE, Bormann CL, Hassun PA, Rocha AM, Motto ELA, Serafini PC and Smith GD. Effects of semen storage and separation techniques on sperm DNA fragmentation. *Fertil Steril* 2010; 94: 2626-2630.

Jager S. Sperm nuclear stability and male infertility. *Arch Androl* 1990; 25: 253-259

Jaroudi K, Al-Hassan S, Al-Sufayan H, Al-Mayman H, Qeba M and Coskun S. Intracytoplasmic sperm injection and conventional in vitro fertilization are complementary techniques in management of unexplained infertility. *J Assist Reprod Gen* 2003; 9: 377-381.

Jégou B, Pineau C and Toppari J (eds). Spermatogenesis in vitro in mammals. In: Assisted Reproductive Technology. Cambridge University Press, Cambridge, UK. 2002. p. 3-5.

Jurema MW, Vieira AD, Bankowski B, Petrella C, Zhao Y, Wallach E and Zacur H. Effect of ejaculatory abstinence period on the pregnancy rate after intrauterine insemination. *Fertil Steril* 2005; 84: 678-681.

Kao SH, Chao HT and Wei YH. Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa. *Mol Hum Reprod* 1998; 4: 657-666.

Kazerooni T, Asadi N, Jadid L, Kazerooni M, Ghanadi A, Ghaffarpasand F, Kazerooni Y and Zolghadr J. Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. *J Assist Reprod Genet* 2009; 26: 591-596.

Khan I, Staessen C and Van den Abbeel E. Time of insemination and its effect on in-vitro fertilization, cleavage and pregnancy rates in GnRH agonist/HMG-stimulated cycles. *Hum Reprod* 1989; 4: 921-926.

- Kilani S, Cooke S and Champman M. Time course of meiotic spindle development in MII oocytes. *Zygote* 2010; 19: 55-62.
- Kosower NS, Katayose H and Yanagimachi R. Thiol-disulfide status and acridine orange fluorescence of mammalian sperm nuclei. *J Androl* 1992; 13: 342-348.
- Kruger TF, Menkveld R, Stander FSH and Lombard CJ. Sperm morphological features as a prognostic factor in IVF. *Fertil Steril* 1986; 46: 1118-1123
- Lachaud C, Tesarik J, Canadas ML and Mendoza C. Apoptosis and necrosis in human ejaculated spermatozoa. *Hum Reprod* 2004; 19: 607-610.
- Larson-Cook KL, Brannian JD, Hansen KA, Kasperon KM, Aamold ET and Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril* 2003; 80: 895–902.
- Lee J, Richburg JH, Younkin SC and Boekelheide K. The Fas System is a key regulator of germ cell apoptosis in the testis. *Endocrinology* 1997; 138: 2081-2088.
- Lewis SEM. Is sperm evaluation useful in predicting human fertility? *Reproduction* 2007; 134: 31-40.
- Liu DY and Baker HWG. Morphology of spermatozoa bound to the zona pellucida of human oocytes that failed to fertilize in vitro. *J Reprod Fertil* 1992; 94: 71-84.
- Lopes S, Sun JG, Jurisicova A, Meriano J and Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in Intracytoplasmic sperm injection. *Fertil Steril* 1998; 69: 528-532.
- Mahi CA and Yanagimachi R. The effect of temperature, osmolarity and hydrogen ion concentration on the activation and the acrosome reaction of golden hamster spermatozoa. *J Reprod Fertil* 1973; 35: 55-66.

Makkar G, Ng EH, Yeung WS, Ho PC. The significance of the ionophore-challenged acrosome reaction in the prediction of successful outcome of controlled ovarian stimulation and intrauterine insemination. *Hum Reprod* 2003; 18: 534-539.

Manicardi GC, Bianchi PG and Pantano S. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biol Reprod* 1995; 52: 864–867.

Mansour RT, Serour MG, Abbas AM, Kamal A, Tawab NA, Aboulghar MA and Serour GI. The impact of spermatozoa preincubation time and spontaneous acrosome reaction in Intracytoplasmic sperm injection: a controlled randomized study. *Fertil Steril* 2008; 90: 584-591.

Marcon L and Boissonneault G. Transient DNA strand breaks during mouse and human spermiogenesis: new insights in stage specificity and link to chromatin remodelling. *Biol Reprod* 2004; 70: 910-918.

Marin-Briggiler CI, Tezon JG, Mirana PV and Vazquez-Levin MH. Effect of incubating human sperm at room temperature on capacitation-related events. *Fertil Steril* 2002; 77: 252-259.

Matsuura R, Takeuchi T and Yoshida A. Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. *Asian J Androl* 2010; 12: 753-759.

McDonnell A. Behind the book: historical note. Available at: <http://www.adriennemcdonnell.com/historical-note>. Accessed: February 6, 2010.

Menéndez S and Marina S. The human spermatozoon. In: An Atlas of Human Gametes and Conceptuses. Veeck LL (Ed). The Parthenon Publishing Group, USA. 1999. p. 25-28.

Menkveld R, Stander FSH, Kotze TJVW, Kruger TF and van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum Reprod* 1990; 5: 586-592.

Menkveld R, Franken DR, Kruger TF, Oehninger S and Hodgen GD. Sperm selection capacity of the human zona pellucida. *Mol Reprod Dev* 1991; 94: 71-84.

Menkveld R, Rhemrev JPT, Franken DR, Vermeiden JPW and Kruger TF. Acrosomal morphology as a novel criterion for male fertility diagnosis: relation with acrosin activity, morphology (strict criteria), and fertilization in vitro. *Fertil Steril* 1996; 65: 637-644.

Morris ID, Ilott S, Dixon L and Brison DR. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Hum Reprod* 2002; 17: 990-998.

Mortimer D. Sperm preparation methods. *J Androl* 2000; 21: 357-366.

Mortimer D and Mortimer SD. Laboratory investigation of the infertile male. In: A Textbook of In Vitro Fertilization and Assisted Reproduction: The Bourn Hall Guide to Clinical and Laboratory Practice. 3rd ed. Brinsden PR (Ed). CRC Press, Oxon, UK. 2005. P. 65-66.

Muratori M, Piomboni P and Baldi E. Functional and ultrastructural features of DNA-fragmented human sperm. *J Androl* 2000; 21: 903-912.

Muratori M, Maggi M and Spinelli S. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *J Androl* 2003; 24: 253-262.

Muratori M, Marchiani S, Maggi M, Forti F and Baldi E. Origin and biological significance of DNA fragmentation in human spermatozoa. *Front Biosci* 2006; 11: 1491-1499.

Muratori M, Tamburrino L, Marchiani S, Guido C, Forti G, Baldi E. Critical aspects of detection of sperm DNA fragmentation by TUNEL/flow cytometry. *Syst Biol Reprod Med* 2010; 56: 277-285.

Nabi A, Khalili MA, Halvaei I and Roodbari F. Prolonged incubation of processed human spermatozoa will increase DNA fragmentation. *Androl* 2014; 46: 374-379.

Nallella KP, Sharma RK, Aziz N and Agarwal A. Significance of sperm characteristics in the evaluation of male infertility. *Fertil Steril* 2006; 88: 629-634.



Nasr-Esfahani MH, Salehi M, Razavi S, Anjomshoa M, Rozbahani S, Moulavi F and Mardani M. Effect of sperm DNA damage and sperm protamine deficiency on fertilization and embryo development post-ICSI. *Reprod Biomed Online* 2005; 11: 198-205.

Oehninger S. Strategies for the infertile man. *Sem Reprod Med* 2001; 19: 231-237.

Oleszczuk K, Giwercman A and Bungum M. Intra-individual variation of the sperm chromatin structure assay DNA fragmentation index in men from infertile couples. *Hum Reprod* 2011; 26: 3244-3248

Oliva R. Protamines and male infertility. *Hum Reprod Update* 2006; 12: 417- 435.

Osheroff JE, Visconti PE, Valenzuela JP, Travis AJ, Alvarez J and Kopf GS. Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. *Mol Hum Reprod* 1999; 1017-1026

Paasch U, Sharma RK and Gupta AK. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol Reprod* 2004; 71: 1828-1837.

Palermo G, Joris H, Devroey P and Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*. 1992; 4: 17-18

Pasqualotto FF, Lara LV and Pasqualotto EB. Capacitation and acrosome reaction. In: Human spermatozoa: maturation, capacitation and abnormalities. Lejeune T and Delvaux P (Eds). Nova Science Publishers, New York, USA. Pp.243-256.

Patrat C, Kaffel A, Delaroche L, Guiber J, Jouannet P, Epelboin S, De Ziegler D, Wolf JP and Fauque P. Optimal timing for oocyte denudation and Intracytoplasmic sperm injection. *Obstet Gynecol Int* 2012; 2012: ID403531.

Petrella C, Hsieh J, Blake E, Thrift K, Zacur H and Zhao Y. Human sperm can survive at room temperature for weeks: measured by motility and viability of sperm maintained under various conditions. *Fertil Steril* 2003; 80: 210.

Pincus G and Enzamm EV. The comparative behaviour of mammalian eggs in vivo and in vitro. *J Exp Med* 1935; 62: 665.

Polge C and Rowson LEA. Fertilizing Capacity of Bull Spermatozoa after Freezing at  $-79^{\circ}\text{C}$ . *Nature* 1952; 169: 626-627

Ramos L, van der Heijden GW, Derijck A, Berden JH, Kremer JAM, van der Vlag J and De Boer P. Incomplete nuclear transformation of human spermatozoa in oligo-asthenoteratospermia: characterization by indirect immunofluorescence of chromatin and thiol status. *Hum Reprod* 2008; 23: 259-270.

Rienzi L, Ubaldi F, Anniballo R, Cerulo G and Greco E. Preincubation of human oocytes may improve fertilization and embryo quality after intracytoplasmic sperm injection. *Hum Reprod* 1998; 13: 1014-1019.

Rougier N, Uriondo H, Papier S, Checa MA, Sueldo C and Sedo CA. Changes in DNA fragmentation during sperm preparation for Intracytoplasmic sperm injection over time. *Fertil Steril* 2013; 100: 69-74.

Royere D, Hamamah S, Nicolle JC and Lansac J. Chromatin alterations induced by freeze-thawing influence the fertilizing ability of human sperm. *Int J Androl* 1991; 14: 328-332.

Sailer BL, Jost LK and Evenson DP. Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the TUNEL assay. *J Androl* 1995; 16: 80-87.

Sakkas D and Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 2010; 93: 1027-1036.

Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG and Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 1999; 4: 31–37.

Sakkas D, Seli E and Bizzaro D. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodeling during spermatogenesis. *Reprod Biomed Online* 2003; 7: 428-432.

Saleh RA, Agarwal A, Nada ES, El-Tonsy MH, Sharma RK and Meyer A. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003; 79 (Suppl3): 1597–1605.

Sathananthan AH, Szell A, Ng SC, Kausche A, Lacham-Kaplan O and Trounson A. Is the acrosome reaction a prerequisite for sperm incorporation after intra-cytoplasmic sperm injection (ICSI)? *Reprod Fertil Dev* 1997; 9: 703-709.

Schill WB, Topfer-Petersen E and Heissler E. The sperm acrosome: functional and clinical aspects. *Hum Reprod* 1988; 139-145.

Schuffner A, Morshedi M, Vaamonde D, Duran EH and Oehninger S. Effect of different incubation conditions on phosphatidylserine externalization and motion parameters of purified fractions of highly motile human spermatozoa. *J Androl* 2002; 23: 194-201.

Schulte RT, Ohi DA, Sigman M and Smith GD. Sperm DNA damage in male infertility: etiologies, assays and outcomes. *J Assist Reprod Genet* 2010; 27: 3-12.

Seli E, Gardner DK, Schoolcraft WB, Moffatt O and Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil Steril* 2004; 82: 378-383.

Seligman J, Kosower NS, Weissenberg R and Shalgi R. Thiol-disulfide status of human sperm proteins. *J Reprod Fertil* 1994; 101: 435-443.

Shafik A, Shafik AA and Shafik I. Sperm DNA fragmentation. *Arch Androl* 2006; 52: 197-208.

Sharma RK and Agarwal A. Sperm quality improvement in cryopreserved human semen. *J Urol* 1996; 156: 1008-1012.

Shekarriz M, DeWire DM, Thomas AJT and Agarwal A. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. *Eur Urol* 1995; 28: 31-45

Shoukir Y, Chardonnens D and Compana A. Blastocyst development from supernumerary embryos after Intracytoplasmic sperm injection: a paternal influence? *Hum Reprod* 1998; 13: 1632-1637.

Shulman A, Hauser R and Lipitz S. Sperm motility is a major determinant of pregnancy outcome following intra-uterine insemination. *J Assist Reprod Genet* 1998; 15: 381-385.

Simon L, Brunborg G, Stevenson M, Luttor D, McManus J and Lewis SEM. Clinical significance of sperm DNA damage in assisted reproduction outcome. *Hum Reprod* 2010; 25: 1594-1608.

Singh NP, Muller CH and Berger RE. Effects of age on DNA double-strand breaks and apoptosis in human sperm. *Fertil Steril* 2003; 80: 1420-1430.

Sinha H and Swedloffn RS. Hormonal and genetic control of germ cell apoptosis in the testis. *Rev Reprod* 1999; 4: 38-47.

Spano M, Cordelli E, Leter G, Lombardo F, Lenzi A and Gandini L. Nuclear chromatin variations in human spermatozoa undergoing swim-up and cryopreservation evaluated by the flow cytometric sperm chromatin structure assay. *Mol Hum Reprod* 1999; 5: 29-37.

Steger K, Pauls K and Klonisch T. Expression of proamine-1 and -2 mRNA during human spermiogenesis. *Mol Hum Reprod* 2000; 6: 219-225.

Sukcharoen N and Keith J. Evaluation of the percentage of sperm motility at 24 h and sperm survival ratio for prediction of in vitro fertilization. *Andrologia* 1996; 28: 203-210.

Sutovsky P. Sperm capacitation, the acrosome reaction and fertilization. In: Reproductive Endocrinology and Infertility: Integrating Modern Clinical and Laboratory Practice. Carrell DT and Petersen CM (Eds). Springer, New York, USA. 2010. pp. 389-442.

Tesarik J, Greco E and Mendoza C. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod* 2004; 19:611–5.

Thijssen A, Klerkx E, Huyser C, Bosmans E, Campo R and Ombelet W. Influence of temperature and sperm preparation on the quality of spermatozoa. *Reprod Biomed Online* 2013; 28: 436-442.

Tomlinson MJ, Moffatt O, Manicardi GC, Bizzaro D, Afnan M and Sakkas D. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. *Hum Reprod* 2001; 169: 2160-2165.

Toro E, Fernandez S, Colomar, A, Casanovas A, Alvarez JG, Lopez-Teijon M and Velilla E. Processing of semen can result in increased sperm DNA fragmentation. *Fertil Steril* 2009; 92: 2109-2112.

Trounson AO and Mohr LR. Human pregnancy following cryopreservation thawing and transfer of an eight-cell embryo. *Nature* 1983; 305: 707–709.

Trounson AO, Mohr LR, Wood C and Leeton JF. Effect of delayed insemination on in-vitro fertilization, culture and transfer of human embryos. *J Reprod Fertil* 1982; 64: 285-294.

Twigg J, Fulton N, Gomez E, Irvine DS and Aitken RJ. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod* 1998; 13:1429–1436.

Van de Velde H, De Vos A, Joris H. Effect of timing on oocyte denudation and micro-injection on survival, fertilization and embryo quality after intracytoplasmic sperm injection. *Hum Reprod* 1998; 13: 3160-3164.

Van der Westerlanken L, Naaktgeboren N, Verburg H, Dieben S and Helmerhorst FM. Conventional in vitro fertilization versus intracytoplasmic sperm injection in patients with borderline semen: a randomized study using sibling oocytes. *Fertil Steril* 2006; 85: 395-400.

Veeck LL. Atlas of the human oocyte and early conceptus. Vol.2. Williams and Wilkins, 1991.

Villarroya S and Scholler R. Lateral diffusion of a human sperm-head antigen during incubation in a capacitation medium and induction of the acrosome reaction in vitro. *J Reprod Fertil* 1987; 80: 545-562.

Villegas J, Kehr K, Soto L, Henkel R, Mishka W and Sanchez R. Reactive oxygen species induce reversible capacitation in human spermatozoa. *Andrologia* 2003; 35: 227-232.

Virro MR, Larson-Cook KL and Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in vitro fertilization and Intracytoplasmic sperm injection cycles. *Fertil Steril* 2004; 81: 1289–1295.

Visconti PE and Kopf GS. Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod* 1998; 59: 1-6.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P and Kopf GS. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Dev* 1995; 121: 1129-1137.

Visconti PE, Ning XP, Fornes MW, Alvarez JG, Stein P and Connors SA. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Dev Biol* 1999; 214: 429-443.

Wang WH Meng L, Hackett RJ, Oldenbourg R and Keefe DL. Rigorous thermal control during intracytoplasmic sperm injection stabilizes the meiotic spindle and improves fertilization and pregnancy rates. *Fertil Steril* 2002; 77: 1274-1277.

Ward WS and Coffey DS. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod* 1991; 44: 569-574.

Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Rep Sci* 2000; 60-61: 481-492.

Wennerholm UB. Cryopreservation of embryos and oocytes: obstetric outcome and health in children. *Hum Reprod* 2000; 15: 18-25.

Williams AC and Ford WC. The role of glucose in supporting motility and capacitation in human spermatozoa. *J Androl* 2001; 22: 680-695.

World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 2010. WHO Press, Geneva, Switzerland.

Wykes SM and Krawetz SA. The structural organization of sperm chromatin. *J Biol Chem* 2003; 278: 29471-29477.

Wyllie AH, Kerr JFR and Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980; 68: 251-306.

Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I and Jabs EW. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *PNAS* 2006; 103: 9601–9606.

Yanagida K, Yazawa H and Katayose H. Influence of oocyte preincubation time on fertilization after intracytoplasmic sperm injection. *Hum Reprod* 1998; 13: 2223-2226.

Yanagimachi R, Yanagimachi H and Rogers BJ. The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol Reprod* 1976; 15: 471-476.

Yavas Y and Selub MR. Intrauterine insemination (IUI) pregnancy outcome is enhanced by shorter intervals from semen collection to sperm wash, from sperm wash to IUI time, and from semen collection to IUI time. *Fertil Steril* 2004; 82: 1638-1647.

Zenzes MT, Belkien L, Bordt J, Kan I, Schneider HPG and Nieschlag E. Cytological investigation of human in vitro fertilization failures. *Fertil Steril* 1985; 43: 883.

Zini A and Libman J. Sperm DNA damage: clinical significance in the era of assisted reproduction. *CMAJ* 2006; 175: 495-500.

Zini A, De Lamirande E and Gagnon C. Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase and catalase-like activities in seminal plasma and spermatozoa. *Int J Androl* 1993; 16: 183-188.

Zini A, Finelli A, Phang D and Jarvi K. Influence of semen processing technique on human sperm DNA integrity. *Urology* 2000; 56: 1081-1084.

Zini A, Bielecki R, Phang D and Zenzes MT. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 2001; 75: 674-677.

Zini A, Meriano J, Kader K, Jarvi K, Laskin CA and Cadesky K. Potential adverse effect of sperm DNA damage on embryo quality after ICSI. *Hum Reprod* 2005; 20: 3476–3480.



## **ADDENDUM I: SEMEN ANALYSIS ESTIMATION**

### **Viscosity**

The viscosity of liquefied semen is measured by the drop method. The length of a thread produced by a drop from a pipette when it is slowly released is estimated.

- Normal: 1cm
- Increased: >1cm

### **Volume**

The volume is measure in a graduated 15mL centrifuge tube.

- Normal: 2-6 mL
- < 2mL: hyperspermia
- >6 mL : hypospermia

### **Motility, forward progression and concentration estimation**

A drop of semen is placed on a clean microscope slide and covered with a coverslip. Ensure that no bubbles form under the slide. Allow the wet preparation to stabilize for about a minute. Assess the wet preparation when it is clear that the semen contents are no longer drifting. The slide is examined under phase contrast microscope (400x magnification) for motility, forward progression and concentration

### Motility

The percentage motile sperm is distinguished from the percentage immotile sperm

### Forward progression

Motility is evaluated on a scale of 0 to 4 (Addapted from Eliasson, 1978)

0	no movement
1	movement - none forward
1+	movement - a few now and then
2	movement - undirected, slow
2+	movement - slowly but directly forward
3-	movement - fast but undirected
3	movement - fast and directly forward
3+	movement - very fast and directly forward
4	movement - extremely fast and directly forward (wave-like)

### Concentration estimation

A drop of semen of the well mixed, undiluted semen is placed onto a clean glass slide and covered with a cover slip. Ensure that no bubbles form under the slide. Allow the wet preparation to stabilize for about a minute. Assess the wet preparation when it is clear that the semen contents are no longer drifting.

Examine the slide under the 400x magnification of the microscope. To determine the sperm concentration, scan the slide and count the number of spermatozoa per field.

After having examined a few fields, you might find that the number of spermatozoa varies greatly per field. This can indicate that the sample is not homogenous and should be mixed again and repeated.

## **Morphology**

For the determination of the percentage of morphologically normal spermatozoa, a thin evenly spread smear is made from the semen sample, taking the sperm concentration into account, and allowed to air dry. The slide is stained with the DQ (addendum II).

The sperm is counted under the 100x oil immersion objective. Normal sperm cells are distinguished from the abnormal sperm cells. The whole spermatozoa (head, neck, midpiece and tail) must be taken into consideration. The head is required to have a smooth oval configuration with a well-defined acrosome, comprising 40-70% of the sperm head. All borderline normal forms are regarded as abnormal.

<4: poor

≥4: normal

## **ADDENDUM II: D-Q STAINING METHOD**

### **MATERIALS**

Hemacolor Staining Set for Microscopy

- Hemacolor solution 1, fixing solution
- Hemacolor solution 2, colour reagent red
- Hemacolor solution 3, colour reagent blue

### **METHOD**

A thin smear is prepared on a glass slide and air dried.

1. Fix the smear for 10 seconds in methonal fixative
2. Stain the slide for 7 seconds in solution 1 (colour reagent red)
3. Stain the slide for 10 seconds in solution 2 (colour reagent blue)
4. Rinse slide by gently dipping it repeatedly into flowing tap water
5. Allow to air-dry

### **MORPHOLOGY EVALUATION**

- Once the slide is air-dried, evaluate the slide under a light microscope
- Under the 10 x objective, find a field with spermatozoa
- Place a drop of immersion oil onto the spot and change the objective to the 100x magnification
- Count 100 sperm cells

## **ADDENDUM III: STANDARD SWIM-UP PREPARATION**

### **MATERIALS:**

- Sperm Washing Medium (SAGE In-Vitro Fertilization Inc., Cooper Surgical Company, Trumbull, CT)
- 3 x test tubes
- Glass slide and cover slip

### **METHOD:**

#### **Semen Collection and Analysis**

- Obtain semen sample by masturbation
- Allow sample to liquefy for 20 minutes
- Estimate the count, motility and forward progression of the sperm

#### **Semen Preparation**

1. Dilute 0.5-1ml semen with 2ml warm sperm preparation media
2. Vortex and centrifuge the sample at 450g for 10 minutes
3. Remove the supernatant and resuspend the pellet again in 2ml of warm sperm preparation medium.
4. Vortex and centrifuge at 450g for 10 minutes
5. Remove the supernatant and gently overlay 0.5ml of sperm preparation medium
6. Place the tube at an angle in the incubator set to 37°C for 60 minutes
7. After 60 minutes, gently remove the 0.5 ml of sperm preparation medium and place in a clean tube
8. Make a wet-preparation slide of the sample and assess as outlined by the WHO criteria (WHO, 2010)

## **ADDENDUM IV: SILSELECT GRADIENT PREPARATION**

### **MATERIALS**

- SilSelect Media (Fertipro, Beernem, Belgium)
- Sperm Washing Medium (SAGE In-Vitro Fertilization Inc., Cooper Surgical Company, Trumbull, CT)

### **METHOD**

#### **Semen collection and analysis**

- Obtain semen sample by masturbation into sterile plastic containers
- Allow fresh sample to liquefy and prepare within one hour of ejaculation
- Make a wet preparation of the sample and evaluate the count, motility and forward progression

#### **Gradient Preparation**

- Discontinuous density gradients can be prepared with either two or three layers  
2 layers: 90% and 45% SilSelect  
3 layers: 90%, 70% and 45%
- To prepare the gradient, 1ml of the SilSelect dilution with the highest density (90%) is placed at the bottom of a sterile conical gradient tube
- Gently overlay with an equal volume of 45% SilSelect if a two-layer gradient is required, or 70% and then 45% SilSelect when a three layer gradient is required
- The gradient is left to equilibrate at 37°C
- Gently layer the semen onto the prepared, warmed gradient
- Centrifuge at 400g for 18 minutes
- Following centrifugation, carefully aspirate the supernatant from the meniscus down towards the 90% SilSelect layer using a small bore-hole pipette, such as a glass pipette. Gently aspirate the top third of the 90% SilSelect layer leaving behind the sperm pellet and those sperm cells that may still be suspended in the remaining 90% SilSelect
- Using a new pipette, remove the pellet from the gradient tube and place into a sterile conical-bottom tube.
- Resuspend the remaining 90% SilSelect media containing sperm with 0.3ml sperm preparation media and flush (by aspirating in and out) the area of the tube where the bottom layer of the gradient was to capture any sperm stuck to the sides of the tube

- Remove the suspension and transfer into the clean tube where the sperm pellet was placed
- Add 2ml of warm sperm preparation medium to the suspension to dilute out the 90% SilSelect
- Centrifuge at 450g for 10 minutes
- Remove the supernatant and resuspend the pellet again in 2ml of warm sperm preparation medium
- Centrifuge at 450g for 10 minutes
- Remove the supernatant and resuspend the pellet in 0.2ml of warm sperm preparation medium

Make a wet preparation slide of the sample and assess count motility and forward progression as outlined by the WHO criteria

## **ADDENDUM V: TUNEL METHOD**

### **MATERIALS**

- 1% HSA/PBS
- 4% formaldehyde/PBS solution
- 0.1% Triton X-100/ PBS
- TUNEL reaction mixture
  - o In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, GmbH, Mannheim, Germany)
- Dabco Anti-fade
- Teflon printed diagnostic slides (21 wells)

### **METHODS**

#### **Sample Preparation**

##### Freezing

- After incubation of each sample at different temperatures, store a portion of the incubated specimen (>30µl) in a pipette tip
- Plunge the pipette tip into liquid nitrogen
- Store samples in a separate student/research liquid nitrogen tank

##### Thawing

- This should be done on the day of TUNEL evaluation
- Thawing samples for 10 minutes at 37°C
- 20 samples can be thawed for analysis

#### **TUNEL procedure**

1. Aliquot 5µl of each sample on one of the wells of the diagnostic slide. Leave well 21 open as a negative control (figure 1).





Figure 1: Teflon Printed diagnostic slide. Red indicates where the negative control is  
(www.2api.com)

2. Allow the slide to air-dry for 60 minutes at room temperature
3. Fixation step: Add 5µl 4% formaldehyde/PBS solution to each well
4. Incubate the slide for 45 minutes at room temperature in a humidified chamber
5. Siphon the excess liquid carefully using tissue paper
  - Siphon from the edges of each spot – the paper should not touch the well
  - Remove as much fluid as possible
  - Use a fresh or uncontaminated part of tissue paper to avoid contamination
6. Washing step: Wash each sperm well with 10µl 1% HSA/PBS and siphon (step 5)
7. Permeabilization step: Add 5µl 0.1% Triton X-100/PBS
8. Incubate at room temperature for 10 minutes in a humidified chamber
  - During this time, thaw label and enzyme solution
9. Siphon (step 5)
10. Perform washing step twice (step 6)

Perform in the dark:

11. Add 5µl of the thawed label solution to well 21 (figure 1)
12. Add the total contents of the thawed enzyme solution to the remaining label solution and mix well
13. Add 5µl of TUNEL reaction mixture to each well (except the negative control)
14. Incubate for 90 minutes at 37°C in a dark, humidified chamber
15. Siphon (step 5)
16. Perform washing step twice (step 6)
17. Add Dabco Anti-fade solution to each well
18. Mount with coverslip – avoid the occurrence of air bubbles
19. Evaluate slide immediately using a fluorescent microscope

## Evaluation

- Count 100 sperm cells per well under 100 x oil immersion objective
- Do not select fields for evaluation on the outer edges of the well
- Interpretation:

- Bright green fluorescent sperm are counted as TUNEL positive
- No fluorescent staining are counted as TUNEL negative
- A faint green colour is considered TUNEL negative

## ADDENDUM VI: CMA<sub>3</sub> METHOD

### MATERIALS

- McIlvaine's Buffer Stock Solutions
  - o Solution 1: Dissolve 3.8484g citric acid (anhydrous) in 200ml distilled water
  - o Solution 2: Dissolve 25.5564g disodium phosphate (anhydrous) in 900ml distilled water
- McIlvaine's Buffer Working Solution (1 Litre)
  - o Dilute 176.5ml Solution with 823.5ml of solution 2
- Chromomycin A<sub>3</sub>
  - o Dissolve 10mg CMA<sub>3</sub> in 1ml ethanol
  - o Store at -20°C in 50µl aliquots
  - o Add 250µl of McIlvaine's buffer to each aliquot before use
- Fixative: Methanol: Acetic acid (3:1)
- Dabco Anti-fade solution

### METHODS:

#### CMA<sub>3</sub> Staining:

1. Prepare smears, and allow air-drying
2. Fix smears for 20 minutes at room temperature in methanol: acetic acid and allow air-drying
3. Stain slides for 20 minutes with 15µl CMA<sub>3</sub> in a dark chamber
4. Rinse slides in McIlvaine's buffer
5. Mount slides with Dabco anti-fade solution
6. Cover slides with coverslips - avoid the occurrence of air bubbles
7. Store slides overnight in the dark room at temperature and evaluate the next morning

#### Evaluation:

8. Evaluate slides are evaluated using a fluorescence microscope
9. Use immersion oil and the 100 x phase contrast objective
10. Count one hundred spermatozoa and evaluate the sperm head for staining quality
  - Bright yellow stained spermatozoa are counted as CMA<sub>3</sub> positive cells
  - Dull yellow stained spermatozoa are counted as CMA<sub>3</sub> negative cells
  - No fluorescent staining sperm cells are counted as CMA<sub>3</sub> negative cells

## **ADDENDUM VII: OOCYTE RETRIEVAL PROTOCOL**

### **METHOD**

- Follicular fluid is aspirated using a long 16 gauge aspiration needle into sterile tubes and sent through to the laboratory.
- The fluid is poured into large petri dishes under the stereomicroscope fitted with a heated stage.
- Follicular fluid must be examined immediately after follicular aspiration and not at 37°C for later examination because red and white blood cells tend to adhere strongly to the cumulus cells.
- If the cumulus is heavily stained with blood then those areas of cumulus can be removed with sterile needles because it is well established that the blood interferes with the fertilization rate and subsequent embryo quality.
- The oocyte-corona-complexes (OCC) are identified, graded (MI vs. MII) and collected with a sterile, rounded, wide-bore glass pipette.
- The OCC are transferred into test tubes (MI and MII) into approximately 2.0ml fresh, warmed flushing medium to wash them of excess blood.
- The OCC are then transferred into Greiner dish with 2.0ml gassed fertilization medium at 37°C and incubated until denuding or insemination time.

## **ADDENDUM VIII: IVF PROCEDURE**

### **Semen Preparation**

- See Addendum II and III

### **Medium preparation**

- Medium is prepared the previous day and place into a CO<sub>2</sub> incubator for equilibration.

### **Aspiration**

- Check suction pump (100-120mmHG)
- Prepare glass polished pipettes for pick up
- Place pick-up tubes in heated block
- Hand theatre medium tube to sister when needed
- Place aspirated follicular fluids in heated block and examine for oocyte-cumulus complexes using a large petri dish on a heated stage of a dissection microscope
- Determine maturity (GV, MI, MII)
- Put the complexes in the pick-up tubes (MI and MII separate)
- When done, rinse all complexes in small petri dish with gassed fertilization medium – check number obtained
- Transfer to 4 well NUNC dish with gassed fertilization medium – maturities separate and not more than 5 complexes per well
- Incubate in the CO<sub>2</sub> incubator until insemination

### **Insemination**

- Inseminate complexes with the correct number/volume of prepared sperm cells according to the sperm count after semen preparation
- Do insemination  $\pm 40$  hours post HCG administration if possible
- Incubate overnight at 37°C, 5% CO<sub>2</sub>
- The next morning, clean oocytes with denuding pipette (Cook, Australia) and check for fertilization

## **ADDENDUM IX: ICSI PROCEDURE**

### **METHOD**

#### **Prior to Injection**

##### *Denuding of oocytes*

- Cumulus cells are removed using hyaluronidase solution and fire drawn, polished glass Pasteur pipettes.
- Corona cells are then removed using denuding pipettes with a stripper pipette attached.

#### **Intracytoplasmic Sperm Injection**

The microinjection procedure is performed on an inverted microscope equipped with:

1. A heated microscope stage (37°C)
2. Two coarse and fine control manipulators
3. Two micro-injectors: one for the holding pipette and the other for the injection pipette

#### ***Procedure***

##### Assembling pipettes

- Insert the holding pipette into the pipette holder
  - o Manually position the pipette using the coarse manipulators so that the angled section is perpendicular to the microscope stage
- Insert the microinjection pipette into the pipette holder
  - o Manually position the pipette so that the angled section is at an angle approximately 20° to the microscope stage
- Ensure that the two pipettes (holding and injection) move in line with each other

##### Microinjection Dish

- Made according to the configuration of the microinjection system and the experience of the technician
- The dish contains an oocyte droplet (flushing medium), spermatozoa (in sperm prep medium) and a PVP droplet.

### Sperm Selection and Immobilization

- Lower the microinjection pipette carefully into the clean PVP droplet
- Repeatedly aspirate and expel PVP medium to coat the inside of the microinjection pipette
- Aspirate a large enough volume (for optimal control) of PVP
- Pipette is lifted and moved to sperm storage droplet
- Selecting sperm cells
  - o Motile sperm cells swim to the outer perimeter of the droplet
  - o Preference is given to morphologically normal and forward progression sperm cells
- Motile sperm cells are aspirated into the injection pipette
- Pipette is lifted and then moved to the clean PVP droplet
- Pipette is lowered to just about the surface in the droplet and the sperm is slowly injected moving up or down so that the sperm tail is transversely positioned to the pipette
- Immobilization
  - o Pipette is lowered onto the section of the tail just below the sidepiece
  - o Pressure and movement is applied with the microinjection and over the sperm cell tail and this destabilizes the sperm cells' membrane and immobilizes the sperm cell
  - o Sperm cell is aspirated and ejected repeatedly to ensure that the sperm cell can be ejected during the injection procedure
  - o Selected immobilized sperm cell is aspirated tail first into the microinjection pipette
  - o The microinjection pipette is lifted and moved to the oocyte droplet

### Oocyte Microinjection

- An oocyte is added to each oocyte droplet in the dish.
- Using the microinjection pipette the oocyte is rotated to locate the polar body at the 12 o'clock or 6 o'clock position
- The holding pipette is lowered and the oocyte held by gentle suction
- The microscope is focused on the oocytes equatorial plane and the internal lumen of the holding pipette
- The microinjection pipette is lowered into the same focus plane at the 3 o'clock position
  - o The plane of the microinjection pipette can be corrected by gently pushing on the ZP

- The sperm cell is carefully brought forward to the point of the microinjection pipette and the pipette is pushed carefully through the ZP and through the oolemma and ooplasm
- The successful penetration of the oolemma is indicated by the ability to aspirate ooplasm into the pipette
- The ooplasm and sperm cell are then carefully ejected into the oocyte and the microinjection pipette is then withdrawn and the oocyte released from the holding pipette.

The whole procedure is repeated for all the oocytes in the microinjection dish



## **ADDENDUM X: EMBRYO GRADING DAY 2 AND 3**

This is a modified version of the Veeck grading (Veeck, 1991)

- Grade 1 Embryo with few blastomeres of any size, severe or complete fragmentation
- Grade 2: Embryo with blastomeres of equal and unequal size, significant cytoplasmic fragmentation
- Grade 3: Embryo with blastomeres of distinctly unequal size, few or no cytoplasmic fragment
- Grade 4: Embryo with blastomeres of equal size; minor cytoplasmic fragments.
- Grade 5: Embryo, with blastomeres of equal size; no cytoplasmic fragments.

## **ADDENDUM XI: BLASTOCYST GRADING**

### **Degree of Expansion and hatching status**

- 1: Early blastocyst, the blastocoel filling more than half the volume of conceptus, but no expansion in overall size as compared to early cleavage stage embryos
- 2: Blastocyst, the blastocoel filling more than half of the volume of conceptus, with slight expansion in overall size and notable thinning of the zona pellucida.
- 3: Full blastocyst, a blastocoel more than 50% of the conceptus volume and overall size fully enlarged with a very thin zona pellucida
- 4: Hatching blastocyst, non-preimplantation genetic diagnosis. The trophectoderm has started to herniate
- 5: Fully hatched blastocyst, non-preimplantation genetic diagnosis. Free blastocyst fully removed from the zona pellucida.
- 6: Hatching or hatched blastocyst, preimplantation genetic diagnosis.

### **Inner cell mass (ICM) grading**

- A Tightly packed, compacted cells
- B Larger loose cells
- C No ICM distinguishable
- D Cells of ICM appear degenerative

### **Trophectoderm grading**

- A Many healthy cells forming a cohesive epithelium
- B Few, but healthy cells, large in size
- C Poor, very large or unevenly distributed cells, may appear as few cells squeezed to the side
- D Cells of the trophectoderm appear degenerative



HUMAN BLASTOCYSTS IN VITRO

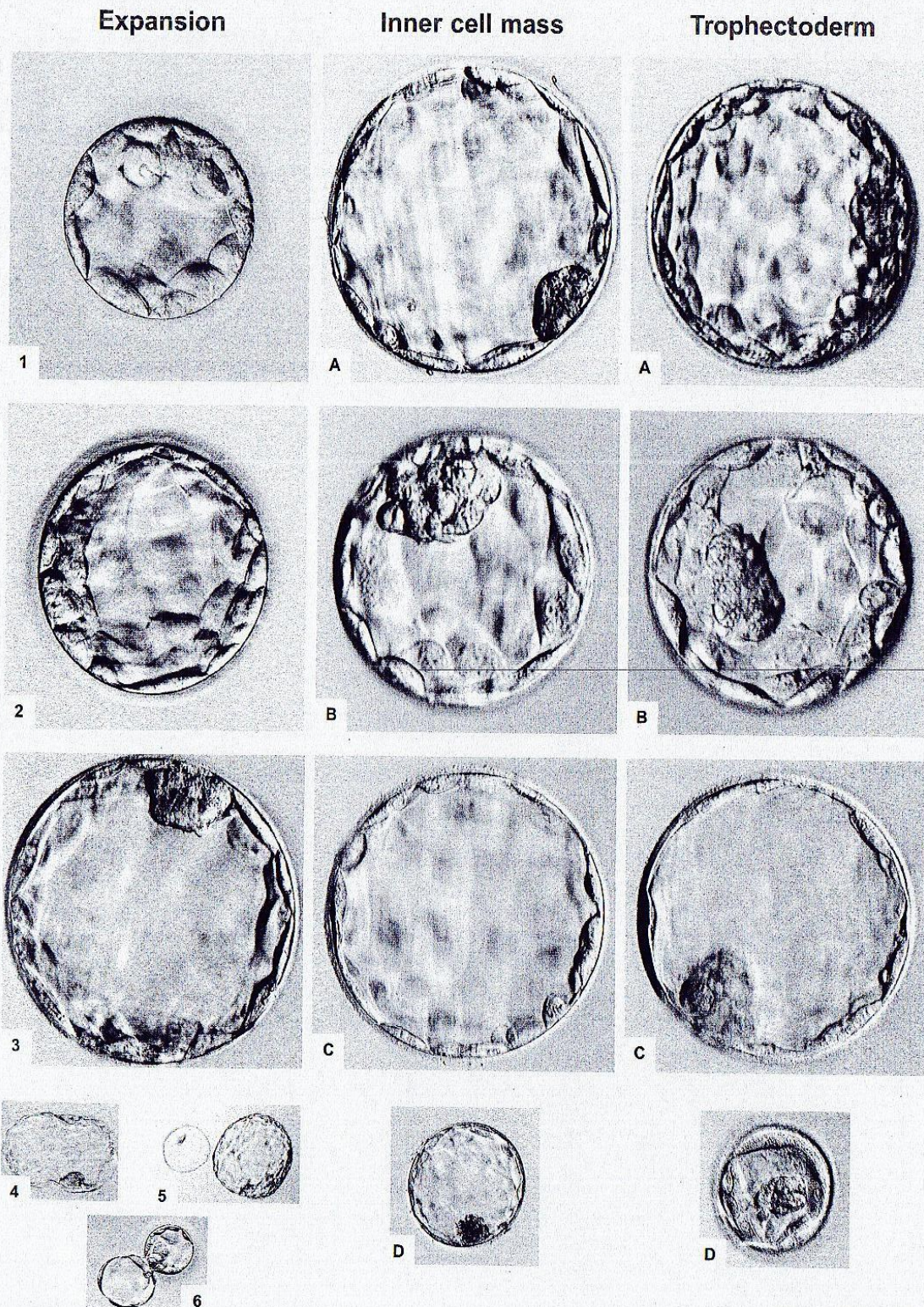


Figure 5.11 Continued

